Structure – function relationships in fructosyltransferase enzymes from *Lactobacillus reuteri* 121

Łukasz Krzysztof Ozimek

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mamie

Preface

Moving to a new country is never an easy task, dealing with a new culture, new customs and a new life. It only took a short while however to settle down and enjoy the Dutch way of living. There were a number of people who helped a lot to get things started in the lab. I would like to especially thank a few people in particular who always found the time to share their invaluable scientific advice with me. To Lubbert, Marc (my promotor and copromotor), Slavko and Sacha (Starcraft/ Warcraft opponents☺), thank you.

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.

Przedmowa

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Chapter 1

General introduction: Fructosyltransferases of lactic acid bacteria

S.A.F.T. van Hijum, S. Kralj, L.K. Ozimek, L. Dijkhuizen, and G.H. van Geel-Schutten

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1. Lactic acid bacteria

Lactic acid bacteria (LAB) are Gram-positive microorganisms, which produce lactic acid as the major end product during the fermentation of carbohydrates. They are found in habitats rich in carbohydrate-containing substrates. Examples of such habitats are mucosal membranes of man and animal (oral cavity, intestine and vagina), plants or material of plant origin, manure, and man-made habitats such as sewage, spoiled food and fermenting food [198]. The LAB group comprises the genera *Lactobacillus, Streptococcus*, *Lactococcus, Leuconostoc*, *Pediococcus, Aerococcus, Alloicoccus, Dolosigranulum, Enterococcus, Globicatella, Lactospaera, Oenococcus, Carnobacterium, Tetragenococcus***,** *Vagococcus* and *Weissella*. Historically, the genera *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* form the core of the LAB group [8,162] (Fig. 1). Members of these genera play an essential role in the fermentation of food (for instance cheeses, milks, breads, wines, pickles and meats) and feed. LAB contribute to the natural preservation of the fermented products by lowering the pH due to the production of lactic acid, and in some cases by the production of antimicrobials. In addition to providing this effective form of natural preservation, they are of influence on the flavor, texture and, frequently, the nutritional attributes of the products [8]. The biochemical processes of LAB, which are of technological importance, are carbohydrate and citrate metabolism, proteolysis, production of antimicrobials and the production of an abundant variety of homo- and heteropolysaccharides [35,39]. There is also a growing interest in LAB and their polysaccharides because of the health benefits (pro- and prebiotics) they confer and various other medical applications.

2. Exopolysaccharides of lactic acid bacteria

Extracellular polysaccharides (exopolysaccharides; EPS) occur widely among bacteria and microalgae and, less frequently, among yeasts and fungi [39,53,131,197]. Some LAB**,** including species of *Lactobacillus***,** are known to produce EPS. These EPSs are large molecules with molecular masses varying between 10^1 to 10^4 kDa, i.e. approximately 50 to 50,000 glycosyl units.

2.1 Heteropolysaccharide synthesis in lactic acid bacteria

Heteropolysaccharides from LAB are composed of a variety of sugar residues, mostly glucose, fructose, galactose and rhamnose. Sometimes charged groups like acetate, phosphate or glycerolphosphate are also present. They are usually produced in low amounts. Heteropolysaccharides are produced by many LAB such as *Lactobacillus sake* [182]*, Lactobacillus casei* [81], *Lactobacillus delbrueckii* spp *bulgaricus* [23,95], *Lactobacillus helveticus*, *Lactobacillus paracasei*, *Lactobacillus acidophilus* [135,136],

Lactobacillus delbrueckii [57], *Lactobacillus rhamnosus* [99,101], *Streptococcus salivarius* ssp. *thermophilus* [4,22,56,103,163], and different *Lactococcus* strains [21,112,180].

Heteropolysaccharides are synthesized at the cytoplasmic membrane by (a series of) glycosyltransferases, using intracellularly formed energy-rich precursors. The biosynthetic pathways of heteropolysaccharides show some similarities with the biosynthesis of the cell-wall components such as lipopolysaccharides, peptidoglycan and teichoic acid. Furthermore, sugar nucleotides serve as precursors in the biosynthesis of different cell-wall components as well as in the biosynthesis of heteropolysaccharides [39,40,76,118,197]. The sugar nucleotides play an essential role in sugar interconversions as well as sugar activation, which is necessary for monosaccharide polymerization. Several enzymes involved in the biosynthesis of heteropolysaccharides, e.g. those involved in the synthesis of the sugar nucleotides, are not necessarily unique to EPS formation, but also play important roles in other metabolic pathways, such as the metabolism of sugars. On the other hand, EPS producing LAB employ specific glycosyltransferases for the assembly of the repeating units [39,197].

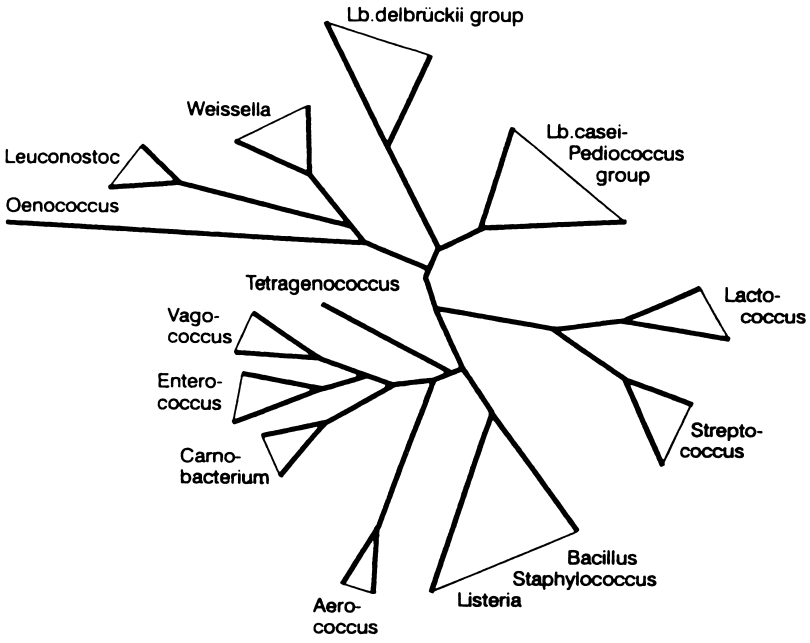

Figure 1. Schematic, unrooted phylogenetic tree of LAB, including some aerobic and facultative anaerobic Gram-positive bacteria of the low G + C subdivision. From [8].

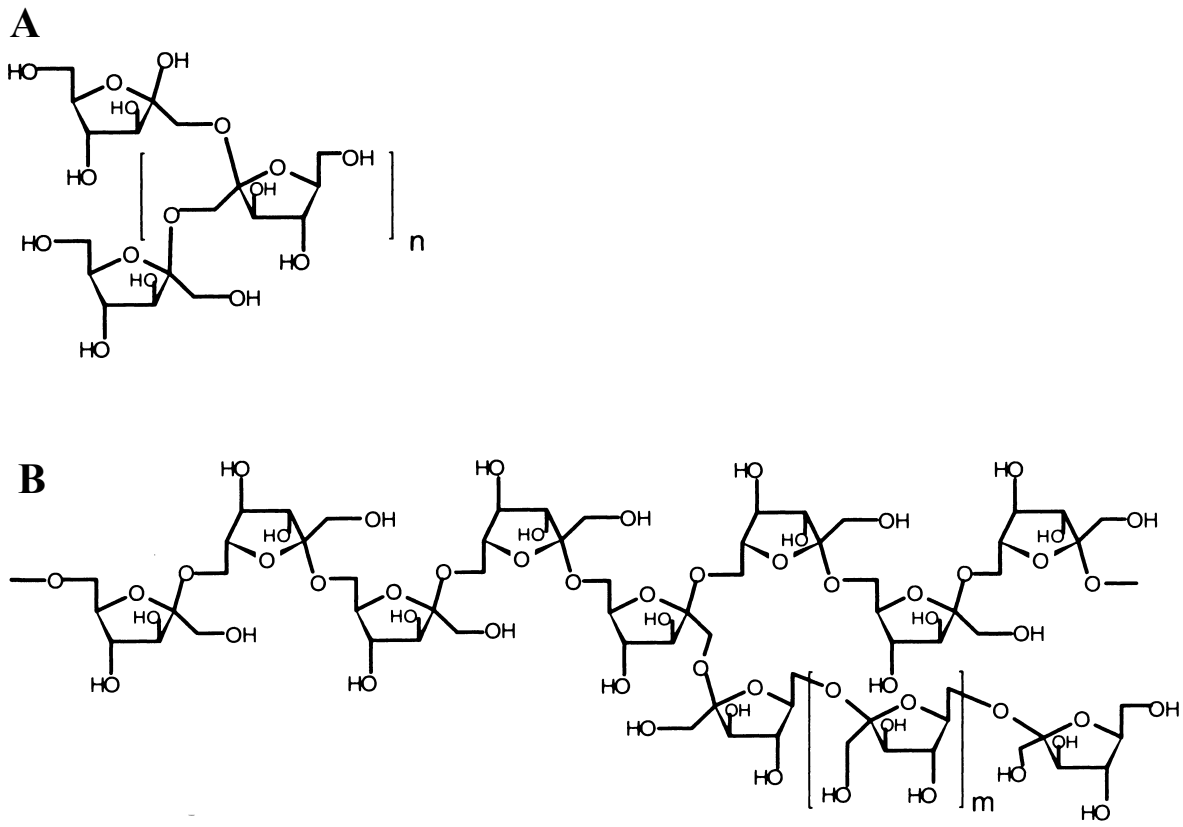

Figure 2. Structures of fructans: A, structure of inulin (where n=20-10000); B, structure of levan (where m=0-2). From [137].

2.2 Homopolysaccharide synthesis of LAB

Homopolysaccharides unlike the heteropolysaccharides of LAB are composed of only one type of glycopyranosyl residue. They consist of either glucose (polymer: α-glucan) or fructose (polymer: fructan) residues with varying binding types and branching degree. They are synthesized by extracellular enzymes of the sucrase type (see below), using a donor molecule, sucrose, and an acceptor molecule, e.g. the growing polymer [137]. The enzymes responsible for the synthesis of glucose polymers have been referred to in the literature as glucosyltransferases (GTFs) or glucansucrases. The sucrases responsible for the production of polymers constituted of fructose are referred to as fructosyltransferases (FTFs). There are two types of fructan synthesizing sucrases known, the inulosucrases, synthesizing inulins (β(2-1) linked fructosyl residues, occasionally containing β(2-6) linked branches; Fig. 2A) and the levansucrases, responsible for the synthesis of levan (β(2-6) linked fructosyl residues, with β(2-1) linked branches; Fig. 2B).

In the presence of suitable acceptor molecules, e.g. maltose, these sucrase enzymes synthesize oligosaccharides (fructo-oligosaccharides or gluco-oligosaccharides) instead of the usually synthesized high molecular weight polymers. These oligosaccharides may contain several types of linkages between the glucose- or fructose moieties depending on the enzyme and conditions used for synthesis [110]. Each enzyme is specific for the types of linkages that are synthesized in their respective polymers.

2.2.1 Glucan synthesis in LAB

Sucrase enzymes only synthesize glucans that are composed of α-glucopyranosyl moieties; the various types of glucan differ in their linkages. Almost all glucans produced by LAB are α-glucans. α-Glucan synthesis has been observed in four different genera of LAB: streptococci, leuconostocs, weissella, and lactobacilli [91,92,109,149,175,185]. In the draft genome sequence of *Oenococcus oeni,* a glucansucrase gene identical to a dextransucrase (*dsr*D) from *Ln. mesenteroides* Lcc4 can be found (http://www.jgi.doe.gov/JGI_microbial/html/index.html). There are examples of β-glucans produced by LAB, for example glucans containing β(1-3)-linked glucose molecules with β(1-2) branches, produced by *Pediococcus* spp. and *Lactobacillus* spp. [44,100]. However these β-glucans are synthesized in the same way, as heteropolysaccharides and sucrase enzymes are not involved in their synthesis.

Roughly, there are five different types of α-glucans synthesized by LAB, dextran α(1-6), mutan α(1-3), alternan α(1-3)/α(1-6), reuteran α(1-4)/α(1-6), and glucan containg α(1-2) branches. A common feature of all dextrans is the abundance of α(1-6) linkages with some branching points at position 2, 3 or 4. Dextrans are produced by for instance *Ln. mesenteroides* strains [21]. Mutans, produced by various streptococci, differ from dextrans in containing a high percentage of α(1-3) linkages; often a water-soluble fraction rich in α(1-6) linkages and a water-insoluble fraction rich in α(1-3) linkages are found [61]. Alternan consists of alternating α(1-6) and α(1-3) linked D-glucopyranosyl units in the main chain and α(1-6-3) branching points and is synthesized by *Ln. mesenteroides* [5]. Reuteran, synthesized by *Lb. reuteri* 121 is a glucan with α(1-6), α(1-4) glycosidic bonds, and α(1-6-4) branching points (*Lb. reuteri* 121) [51,89]. The α(1-2) glucan is produced by two different *Ln. mesenteroides* strains: NRRL-B1299 and a mutant strain (R510) of NRRL B-1355 [17,153].

Glucans exist in large varieties due to differences that occur in the type of linkages, degree and type of branching, length of the glucan chains, molecular weight**,** and conformation of the polymers. As a result, there are large variations in solubility and other physical characteristics of the α-glucans [109]. Many factors, including the growth media, temperature, incubation time, sucrose concentration used, and the presence of polysaccharide degrading enzymes**,** influence the molecular weight, structure, and physical characteristics of the polymers synthesized [78,109].

2.2.2 Fructan synthesis by LAB

Limited information is available on levan production by LAB (Table 1): it has only been reported for streptococci [20,64,151], *Ln. mesenteroides* [138], *Lb. reuteri* 121 [51,186] and *Lb. sanfranciscensis* [84]. Also *Lb. frumenti, Lb. pontis, Lb. panis, Weissela confusa* and *Lb. sanfranciscensis* were found to produce fructans, but the fructan binding types have not been determined [83,87,175,176]. Levan production has been studied in

most detail in non-LAB, *G. diazotrophicus* [66], *Zymomonas mobilis* [93,156], and *Bacillus* sp. [98,127,167] (See also Table 2).

Table 1. Characteristics of FTF enzymes of LAB and their fructan products.

Strain	Fructan	Fructan size (Da)	Oligo	M_r (kDa)	Gene	Reference
Lactobacillus reuteri 121	β(2-6)	97% 2×10^4 3% 3-4$\times10^6$	-	90	*lev*	[186,187]
Lactobacillus reuteri 121	β(2-1)	> 1×10^7	K	90	*inu*	[189]
Lactobacillus sanfranciscensis	fructan$^{\Phi}$	n.d.	K, N	95	*levS*-	[83,87]
Leuconostoc citreum CW28	> 95% β(2-1)	n.d.	n.d.	170	*islA*	[120,121]
Leuconostoc mesenteroides B-512 FMC	levan	n.d.	K, N, P	51.7		[77,154]
Streptococcus mutans AHT	inulin$^{\Phi}$	n.d.	n.d.	-	-	[46]
Streptococcus mutans BHT	90% β(2-1) 10% β(2-1-6)	n.d.	n.d.	-	-	[46]
Streptococcus mutans GS-5	inulin$^{\Phi}$	6-9$\times10^7$	-	88	*ftf*	[69,144]
Streptococcus mutans Ingbritt	inulin$^{\#}$	n.d.	n.d.	70-94	-	[1]
Streptococcus mutans JC1	95% β(2-1) 5% β(2-1-6)	n.d.	n.d.	-	-	[46]
Streptococcus mutans JC2	94% β(2-1) 6% β(2-1-6)	> 2×10^6	n.d.	-	-	[20,139]
Streptococcus mutans OMZ 176	levan$^{\forall}$	n.d.	n.d.	-	-	[33]
Streptococcus salivarius ATCC 13419	levan$^{\Phi}$	2×10^7	n.d.	-	-	[116]
Streptococcus salivarius ATCC 25975	70% β(2-6) 30% β(2-1)	2-10$\times10^7$	-	100	*ftf*	[46]
Streptococcus salivarius HHT	90% β(2-6) 10% β(2-1-6)	n.d.	n.d.	-	-	[46]
Streptococcus salivarius S1	86% β(2-6) 14% β(2-1-6)	n.d.	-	-	-	[64]
Streptococcus salivarius SS2	70% β(2-6) 30% β(2-1)	n.d.	n.d.	-	-	[151]

Binding types identified using mouse antibodies raised against plant inulin polymers.

Φ The binding types of these fructans have not been reported, or it remains unclear how binding types have been determined.

∀ Binding types determined by partial fructan hydrolysis and comparison with inulin and levan standards on TLC.

n.d. Not determined.

K 1-kestose

N nystose

P 1,1,1-kestopentaose

Table 2. Overview of non-LAB bacterial FTFs identified.

Strain	Fructan	Fructan size (Da)	Oligo	M_r (kDa)	Gene	Reference
Arthrobacter sp. K-1	levan$^{\Phi}$	n.d.	n.d.	63	*bff*	ab062134§
Bacillus amyloliquefaciens ATCC 23350	levan$^{\Phi}$	n.d.	n.d.	50	-	[102]
Bacillus amyloliquefaciens ATCC 23844	levan$^{\Phi}$	n.d.	n.d.	50	*sacB*	[171]
Bacillus circulans	levan$^{\Phi}$	n.d.	n.d.	52	-	[127]
Bacillus natto	90% β(2-6) 10% β(2-1-6)	2.5×10^6	-	n.d.	-	[73]
Bacillus subtilis BS5C4	levan$^{\Phi}$	$> 1\times10^7$	-	50	-	[30]
Bacillus subtilis QB112	levan$^{\Phi}$	n.d.	n.d.	50	*sacU*	[28]
Bacillus subtilis var. *saccharolyticus*	levan$^{\Phi}$	2×10^4	n.d.	n.d.	-	[167]
Bacillus subtilis W168	levan$^{\Phi}$	n.d.	n.d.	52	*sacB*	[161]
Clostridium acetobutylicum	levan$^{\Phi}$	n.d.	n.d.	53	*ftf*	[117]
Geobacillus stearothermophilus ATCC 12980	levan$^{\Phi}$	n.d.	n.d.	50	*surB*	[98]
Paenibacillus polymyxa CF43	levan$^{\Phi}$	$> 5\times10^6$	kestose$^{\Psi}$	52	*sacB*	[15]
Paenibacillus polymyxa NRRL B-18475	88% β(2-6) 12% β(2-1-6)	2×10^6	-	52	-	[62,63]
Actinomyces naeslundii WVU45	levan$^{\Phi}$	n.d.	-	68	*ftf*	[14]
Actinomyces viscosus T14	>50% β(2-6)$^{\#}$ <50% β(2-1)$^{\#}$	5×10^7	-	240*	-	[124]
Aerobacter levanicum	levan$^{\Phi}$	5×10^7	n.d.	22	-	[45]
Erwinia amylovora Ea7 / 74	levan$^{\Phi}$	n.d.	n.d.	44	*lsc*	[52,58]
Gluconacetobacter diazotrophicus SRT4	levan$^{\Phi}$	$> 2\times10^6$	1-kestose	58	*lsdA*	[6,66]
Gluconacetobacter xylinum NCI 1005	β(2-6)	n.d.	n.d.	47	*lsxA*	[165]
Pseudomonas syringae pv. glycinea PG4180	levan$^{\Phi}$	n.d.	-	44	*lsc/B/C*	[65]
pv. phaseolicola NCPPB 1321	levan$^{\Phi}$	$1\text{-}100\times10^5$	-	44	*lsc*	[67,68]
Pseudomonas aurantiaca	levan$^{\Phi}$	n.d.	n.d.	47	*lscA*	af306513§
Rahnella aquatilis ATCC 33071	β(2-6)	n.d.	-	43	*lsrA*	[147]
Rahnella aquatilis JCM-1683	levan$^{\Phi}$	n.d.	n.d.	62	-	[119]
Zymomonas mobilis ATCC 10988 / ZM1	β(2-6)	n.d.	1-kestose	47	*levU*	[156,157]
Zymomonas mobilis IFO 13756/Z6	levan$^{\Phi}$	n.d.	kestose	56	*sucE2*	[93,199]
Zymomonas mobilis NRRL B806	levan$^{\Phi}$	n.d.	n.d.	47	*sacB*	[60]

* M_r of this multi-subunit enzyme determined by gel filtration.

\# Binding types identified by mouse antibodies raised against plant inulin polymers.

Φ The binding types of these fructans have not been reported or or it remains unclear how binding types have been determined.

Ψ Minor amounts detected.

§ Genbank accession numbers.

n.d. Not determined.

Inulin production by LAB has only been observed in some cariogenic *S. mutans* and *Streptococcus salivarius* strains [46,139,148], *Ln. citreum* [121], and *Lb. reuteri* (Table 1) [188,189].

Fructan production has been reported for a large variety of non-LAB Gram-positive bacteria and Gram-negative bacteria, the latter involving a larger variety of bacterial species (Table 2). The sizes of the fructans produced (if determined) show a large variation from 2×10^4 to 5×10^7 Da. In some cases, the molecular weight of the fructan produced is dependent on growth/incubation conditions, e.g. the temperature, salinity, and sucrose concentration used [13,67,168,169].

3. Physiological functions of exopolysaccharides in LAB

The physiological roles of exopolysaccharides in (lactic acid) bacteria have not been clearly established, and are probably diverse and complex. The polymers may render protection to microbial cells in their natural environment, against desiccation, phagocytosis and phage attack, antibiotics or toxic compounds (e.g. toxic metal ions, sulfur dioxide, ethanol), predation by protozoans, osmotic stress, alternatively, they may also play a role in adhesion of cells to solid surfaces and biofilm formation, and also in cellular recognition (e.g. via binding to a lectin) [21]. Glucans and fructans may also contribute to the provision of reduced oxygen tension and participate in the uptake of metal ions [21].

Limited information is available about homopolysaccharide synthesis by LAB. Most attention has been focussed on oral streptococci such as *S. mutans* and *S. sanguis* strains [46,139,148] because of their clearly established role in dental caries formation [10]. Both glucans and fructans (see below) formed by oral streptococci apparently have major influences on the formation of dental plaque. They are involved in adherence of bacteria to each other and to the tooth surface, modulating diffusion of substances through plaque and serving as extracellular energy reserves [140]. Other examples of fructans that function as adhesive agents and facilitate interactions between bacteria and their hosts are (i) levan production by the sugar cane plant root invading Gram-negative *G. diazotrophicus* [66] and (ii) the persistence and virulence of *Actinomyces naeslundii* and *Actinomyces viscosus* in the mammalian oral cavity [14].

Several bacilli species (Table 2) and bacterial strains within oral biofilms accumulate fructans as extracellular energy storage form, enabling the organism to store carbohydrates (e.g. dietary sucrose) in a soluble form, which is inaccessible to organisms that lack fructan degrading activities [21,55].

Combinations of biosynthetic and degrading enzyme(s) have been reported in *Lb. plantarum* [113], *B. subtilis* [126], *Paenibacillus polymyxa* [16], *Z. mobilis* [60], several streptococci [31,32,42,125], and *S. mutans* [18,144]. The branched levan produced by *B. subtilis* can be completely degraded by an endolevanase activity, which is associated with the cytoplasmic membrane and an exolevanase activity from the levansucrase itself [126].

The soil bacterium *P. polymyxa* can utilize sucrose to synthesize a high molecular weight levan. In addition, it can also degrade both fructans of the levan and inulin type [62]. Recently, the molecular structure of the *Lb. plantarum* sucrose utilization locus was published, providing *Lactobacillus* genes to utilize various sugars including sucrose and raffinose. The locus showed to be highly similar to the plasmid located sucrose utilization locus of a *Pediococcus* strain, which is normally not able to catabolize sucrose [113]. Both loci contained sucrose degradative enzymes belonging to the glycoside hydrolase (GH) family 32 (see below). Dextran degrading enzymes (dextranases; Dex) of streptococci hydrolyze the glucans of the plaque bacteria (e.g. *S. mutans* and *S. sobrinus*), produced as potential storage carbohydrates and for adherence to the tooth surface [31,32,42,72,125].

4. Fructan synthesizing sucrases (FTFs)

Bacterial fructans are synthesized by FTFs, one of two classes of sucrase enzymes. These extracellular enzymes cleave the glycosidic bond of their substrate sucrose (and in some cases also raffinose) and use the energy released to couple a fructosyl unit to (1) a growing fructan (polyfructose) chain, (2) to sucrose, (3) to water (hydrolysis), or (4) to other acceptors (various mono- and disaccharides [25]). Bacterial inulin is synthesized by inulosucrase (sucrose: 2,1-β-D-fructan 1-β-D fructosyltransferase; E.C. 2.4.1.9; reaction 1) and bacterial levan is synthesized by levansucrase (sucrose: 2,6-β-D-fructan 6-β-D fructosyltransferase; E.C. 2.4.1.10; reaction 2), according to the following reactions:

(1) sucrose + $(2,1\text{-}\beta\text{-D-fructosyl})_n$ → D-glucose + $(2,1\text{-}\beta\text{-D-fructosyl})_{n+1}$

(2) sucrose + $(2,6\text{-}\beta\text{-D-fructosyl})_n$ → D-glucose + $(2,6\text{-}\beta\text{-D-fructosyl})_{n+1}$

Because sucrose is used as acceptor in the initial priming reaction, bacterial fructans contain a non-reducing glucose unit at the end of the chain [50].

Within the LAB, FTF enzymes have been found in *S. salivarius* [54], *S. mutans* [148], *Ln. citreum* [120,121], *W. confusa* [175], *Lb. reuteri* [186,187,189] and *Bacillus* sp. 217C-11 [196]. Levansucrase (but not inulosucrase) enzymes are also found in non-LAB bacteria, e.g. *Z. mobilis* [60,156], *Erwinia amylovora* [52,58], *G. diazotrophicus* [6], *Bacillus polymyxa* [15], *Bacillus amyloliquefaciens* [171], *Bacillus stearothermophilus* [98], and *B. subtilis* [161]. At the amino acid level, the levansucrases of Gram-positive and those of Gram-negative bacteria show low similarity (about 20%; results not shown), which is also reflected by the relatively large phylogenetic distance between groups I and II (Fig. 3).

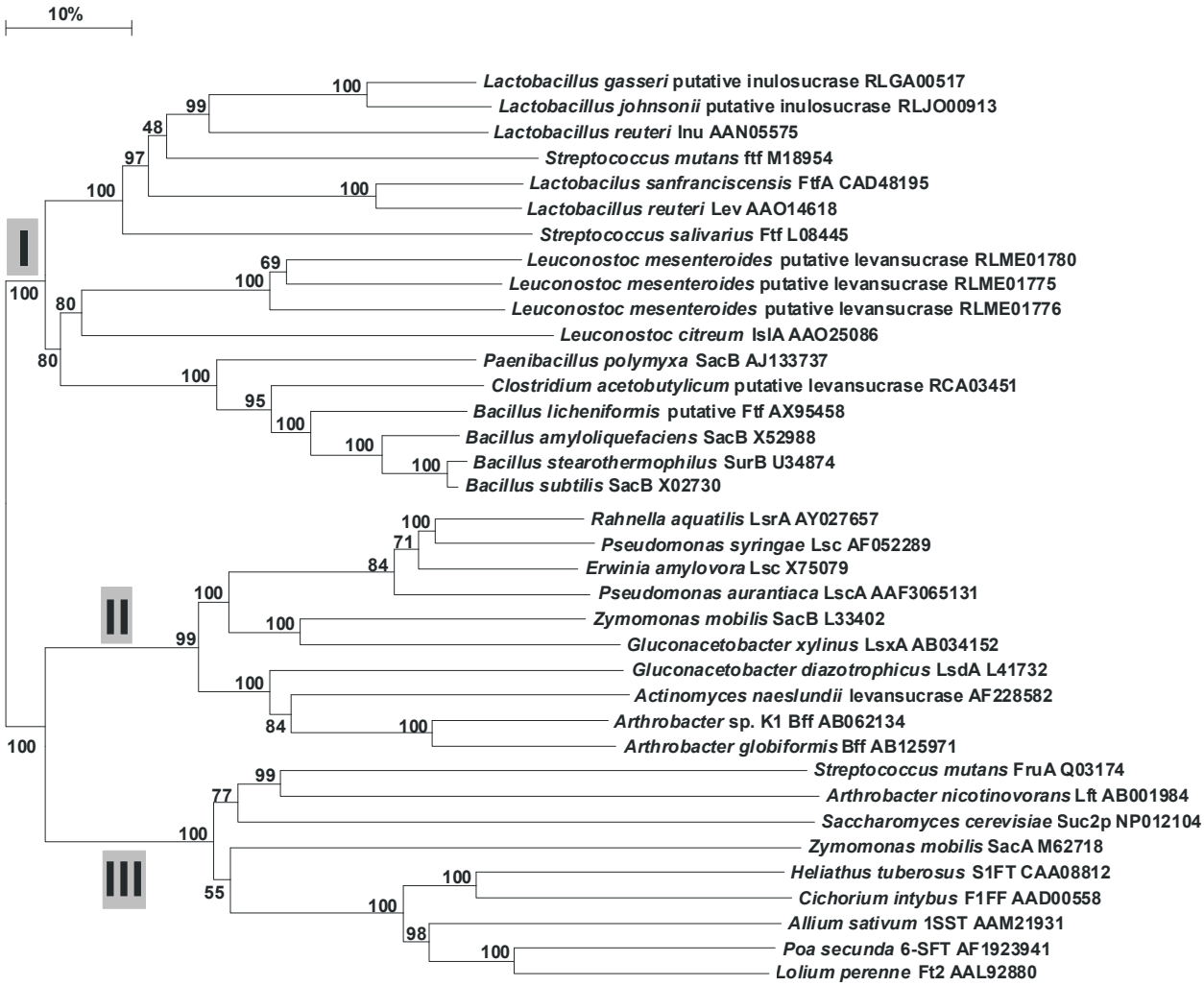

Figure 3. Unrooted phylogenetic tree of FTFs and some invertases from bacteria, plants, and fungi. The bar indicates 10% difference at the amino acid level. Alignments were made (using the complete amino acid sequences) in ClustalW 1.74 [173], a gap opening penalty of 30, and a gap extension penalty of 0.5. Dendrogram construction was done with TreeCon 1.3b [181] using the neighbour joining method with no correction for distance estimation. Bootstrap values (in percentage) are indicated at the branching points. The number of sequences was reduced by removing the following sequences that clustered closely to: (a) X02730: *Nostoc punctiforme* putative levansucrase RNPU00315, *Bacillus subtilis* SacN AY15036; (b) X52988: *Oryza sativa* putative levansucrase ROS01311, *Klebsiella pneumoniae* putative levansucrase RKP01110, *Leishmania major* levansucrase AL160371, *Fibrobacter succinogenes* putative Ftf TIGR59374; (c) RCA03451: *Clostridium acetobutylicum* putative levansucrase AAK79737; (d) RLME01776: *Leuconostoc mesenteroides* RLME01777; (e) RLME01780: *Leuconostic mesenteroides* RLME01781; (f) M18954: *Streptococcus mutans* UA159 RMN00084; (g) L33402: *Zymomonas mobilis* LevU AF081588, *Zymomonas mobilis* SucE2 D17524; (h) AY027657: *Rahnella aquatilis* LsrA U91484; (i) AF052289: *Pseudomonas syringae* LscA AF037443, *Pseudomonas syringae* LscC AF346402, *Pseudomonas syringae* LscB AF345638. The tree is divided into groups: (i) FTFs from Gram-positive bacteria, (ii) FTFs from Gram-negative bacteria with the following exceptions: *Arthrobacter* Bff, *Arthrobacter globinoformis* Bff, and *Actinomyces naeslundii* Ftf from Gram-positive bacteria, and (iii) plant FTFs and invertases / fructan degrading enzymes from various origins.

FTF enzymes present in the Gram-positive bacteria *Arthrobacter* and *Actinomyces* clearly cluster in the Gram-negative group (Fig. 3), which may indicate transfer of these genes from Gram-negative bacteria. The molecular masses of most bacterial levansucrases (and inulosucrases) vary between 50 and 100 kDa [148,161,164,187]. A value of 140 kDa, however, has been reported for the enzyme of *S. salivarius* [121]. The levansucrases of both *S. salivarius* and *Lb. reuteri* appear to be bound to the cell wall. Part of the enzyme is released into the culture medium in the presence of sucrose [107,184,186]. Most of the research on levansucrases has been performed on enzymes from *Zymomonas, Bacillus* and, to a lesser extent, *Gluconacetobacter* species.

5. FTF enzymes from plants and fungi

Fructan biosynthesis in plants and fungi involves a set of enzymes clearly different from their bacterial counterparts. The fructan biosynthetic enzymes in plants are evolutionarily related to invertases, enzymes that hydrolyze sucrose, belonging to family GH32 [183]. A general model of fructan biosynthesis from sucrose in plants was proposed based on the activity of heterologously produced FTFs [195]. Sucrose:sucrose 1-fructosyltransferase (1-SST) initiates *de novo* fructan synthesis by catalyzing the transfer of a fructosyl residue of sucrose to another sucrose molecule, resulting in 1-kestose (precursor for levan and inulin synthesis) and glucose. A second enzyme, fructan: fructan 1-fructosyltransferase (1-FFT), transfers fructosyl residues from one fructan (degree of polymerization; DP $\geq$ 3) to another fructan molecule [195]. The resulting plant inulins are relatively small polymers usually consisting of 20 to 50 fructosyl units per fructan molecule (resulting in fructans with M_r's of 3,000 to 10,000). Both 1-SST and 1-FFT enzymes show unusual kinetics: these enzymes cannot be saturated by their substrate sucrose [82]. Their amino acid sequences are significantly different from the FTFs from Gram-positive bacteria (Fig. 3).

Genes encoding extracellularly located FTFs (belonging to family GH32) have recently been identified in the fungi *Aspergillus sydowi* [70], *Aspergillus niger* [94], *Aspergillus foetidus* [134], and *Penicillium roquefortii* [75]. These genes share a higher sequence homology with plant 1-SST than with bacterial FTFs. Expression of these fungal genes in heterologous hosts showed that these enzymes produced 1-kestose from sucrose [70,94,134]. The fungal enzymes as well as plant 1-SST enzymes thus synthesize identical products (1-kestose). However, a further comparison of the biochemical properties of fungal FTFs, plant FTFs, and bacterial FTFs remains to be carried out.

6. Reaction mechanism of bacterial FTF enzymes

A schematic representation of the various FTF catalyzed reactions is presented in Fig. 4. In the initial reaction of FTFs, the fructose of a sucrose molecule (Fig. 4, ES to EF

step) is coupled by the enzyme to another non-reducing fructose with a free primary alcohol at position C-2, acting as an acceptor substrate, e.g. sucrose, raffinose, or a fructan molecule (Fig. 4A) [41,137]. This is also referred to as the priming reaction. In subsequent steps, the enzyme elongates the primer (in cycles depicted in Fig. 4).

The fructosyl transfer from sucrose (or raffinose) to a number of acceptors other than fructan polymer is catalyzed by all known bacterial FTFs (EF + A in Fig. 4). Examples of possible acceptors are: water (hydrolysis of sucrose), sucrose or raffinose (yielding a tri- or tetrasaccharide, respectively), short chain acylalcohols, various mono- and disaccharides [25], and sorbitol [127]. For the *B. subtilis* levansucrase it was found that the enzyme can use monosaccharides (D-glucose, D-mannose, D-xylose, and D-galactose) as acceptors to produce various di-, tri-, and tetrasaccharides [170]. Four effective acceptors for *Bacillus* sp. levansucrase were studied by Kim *et al.* [80]. High yields of fructosylated acceptor products (26-30% w/w) were accounted for: sucrose, maltose, cellobiose, and lactose. For each acceptor tested, the donor to acceptor concentration ratio optimal for product formation by the enzyme was determined. Using these acceptors, products of a degree of polymerization (DP) 2-7 were synthesized in successive transfer reactions. The enzyme was unable to transfer fructosyl units to sugar alcohols such as D-inositol, D-sorbitol and D-xylitol. The ability of *Lb. sanfranciscensis* levansucrase to use raffinose, maltotriose, maltose, xylose, or raffinose as fructosyl-acceptors leading to formation of range of heterooligosaccharides has been reported recently by Tiekling *et al.* [176]. Additionally, HPLC analysis of the reaction products of this levansucrase with 0.4 M raffinose as fructosyl donor and acceptor revealed presence of tetra-, penta-, and hexasaccharides (GalGF2 – GalGF4) in addition to melibiose (GalG), raffinose (GalGF), kestose (GFF), and nystose (GFFF) proving ability of the enzyme to use raffinose not only as acceptor but also donor of fructosyl moieties.

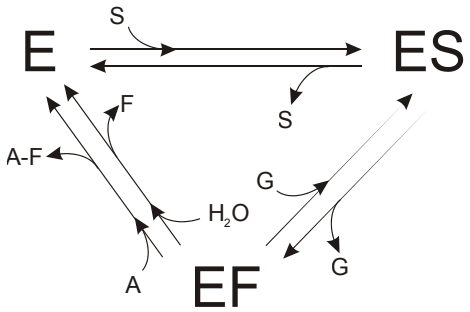

Figure 4. Transfructosylation reaction of FTF enzymes. E, S, G, F, and A represent enzyme, sucrose, glucose, fructose, and fructosyl acceptor, respectively.

A detailed biochemical characterization of the FTF enzyme reactions is complicated by the fact that FTFs generate new fructan molecules, which in turn can be used as acceptor substrates. Accordingly, a multiple-chain-elongation mechanism in which the fructosyl residues are added randomly to all molecules of the fructan acceptors has been proposed [30]. The nature of the fructosyl acceptor (except water) thus changes as the

reaction proceeds (see also Fig. 4; many reactions are reversible). Kinetic and chemical studies of the levansucrase of *B. subtilis* further suggest that each fructosyl unit is added one at a time onto an acceptor molecule [30]. Low molecular-weight fructans accelerate the rate of the polysaccharide formation and increase the fructan to free fructose ratio [47,188]. Most FTF enzymes follow Michaelis-Menten type of kinetics for the hydrolysis and transferase reactions. Exceptions to this rule are the *Lb. reuteri* inulosucrase and levansucrase enzymes, and *Lb. sanfranciscensis* levansucrase which cannot be saturated by their substrate sucrose (in case of the *Lb. reuteri* levansucrase only at 50 °C) [174,187,188]. This phenomenon has also been observed for some plant enzymes that synthesize inulin polymers [82].

A two-step mechanism has been proposed for catalysis by FTF enzymes. Bacterial FTF enzymes belong to glycoside hydrolase family GH68, with an acidic group and a nucleophilic group essential for transfructosylation [152]. Quenching the levansucrase of *B. subtilis* with its substrate sucrose yielded a stabilized enzyme-fructosyl complex. A β-carboxy group of an Asp amino acid residue subsequently was identified as the nucleophilic group binding to the fructosyl group [24]. Information about the position and identity of this Asp residue, and other catalytically important amino acids, recently has become available following the elucidation of the 3D crystal structure of the *B. subtilis* levansucrase (see below) [106]. The *B. subtilis* levansucrase amino acids Asp86, Glu342, and Asp247 provide three conserved acidic side-chains in the enzyme central pocket and play an essential role in catalysis.

Several independent studies revealed that transglycosylation and hydrolysis reactions could be modulated separately by mutagenesis or even by changing reaction conditions. Selective inhibition of transglycosylation activity of *Z. mobilis* levansucrase has been reported by Senthilkumar *et al.* [146]. Three single serine substitutions for cysteine residues decreased sucrose-hydrolysis activity of about 50% and almost completely abolished levan forming activity. Point mutations in “sucrose binding box” in levansucrases of *B. subtilis* and *Z. mobilis* caused significant changes in transglycosylation efficiency of the enzymes (see below). Transglycosylation vs. hydrolysis ratio could be also changed by immobilization of the enzyme on hydroxyapatite and by this mimicking *in vivo* conditions, where levansucrases are attached to cell wall of bacteria or tooth surfaces. Activity of immobilized *B. subtilis* levansucrase was directed mainly toward its polymerizing activity [27].

Based on the observation that the polymerizing activity of the enzyme could be modulated separately from the oligomerization activity a reaction mechanism involving two active sites has been proposed for the *S. mutans* FTF [160]. Recent 3D structural information for the *B. subtilis* levansucrase, however, provides evidence for only a single active site (see below) [106].

7. Structural aspects of FTF enzymes

Although the reactions performed by FTFs and GTFs are similar with respect to the use of sucrose as donor- and acceptor substrate, the proteins involved do not share sequence similarity and/or overall structure. Based on amino acid sequence alignments, bacterial levansucrases (E.C. 2.4.1.10) and inulosucrases (E.C. 2.4.1.9) belong to GH68 family. A core region has been identified that is shared between all family GH68 members (website: http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html; see below) [34].

No structural information is available about LAB FTF enzymes. Recently, high-resolution crystal structures of the *B. subtilis* (non-LAB) SacB levansucrase (at 1.5 Å) and a sucrose-bound inactive mutant of the same enzyme (at 2.1 Å) have been described. The structure shows a rare 5-fold β-propeller topology with a deep, negatively charged central pocket [106]. The central pocket is composed mostly of residues belonging to highly conserved sequence motifs, including invariant acidic residues Asp86, Asp247, and Glu342. These residues form the catalytic triad (nucleophile, transition state stabilizer and acid/base catalyst, respectively) as proven by the mutational analysis of these residues, and their equivalents in *Lb. reuteri* Inu and Lev enzymes [106,123] (see also Chapter 2). Also recently, 3D structure has been reported for the levansucrase (LsdA) enzyme of the Gram-negative, non-LAB *G. diazotrophicus* [104]. This enzyme diplays the same five-bladed β-propeller architecture in which the catalytic residues are superimposable with their equivalents in the *B. subtilis* levansucrase.

Based on deduced amino acid sequences, the overall FTF structure can be divided into 4 regions (Fig. 5): (i) a signal peptide, (ii) an N-terminal stretch of varying length, (iii) a conserved catalytic core of about 500 amino acids, and (iv) a C-terminal stretch of varying length, in some cases with a cell-wall binding domain (LPXTG; see below). Characteristics for each region are discussed below.

7.1. The signal peptide and N-terminal variable domain

Most bacterial FTFs are extracellular enzymes and contain an N-terminal signal sequence that targets these enzymes for secretion (Fig. 5). The signal-peptide containing precursor is cleaved upon secretion of fructosyltransferases by Gram-positive bacteria [14,15,98,132,141,161,171,187]. With exception of the *G. diazotrophicus* LsdA, FTF enzymes of Gram-negative origin are secreted by signal-peptide-independent pathways [7,52,68,156,158,165]. The N-terminal domain (Fig. 5) varies in size between the FTFs and no function has yet been assigned to this domain.

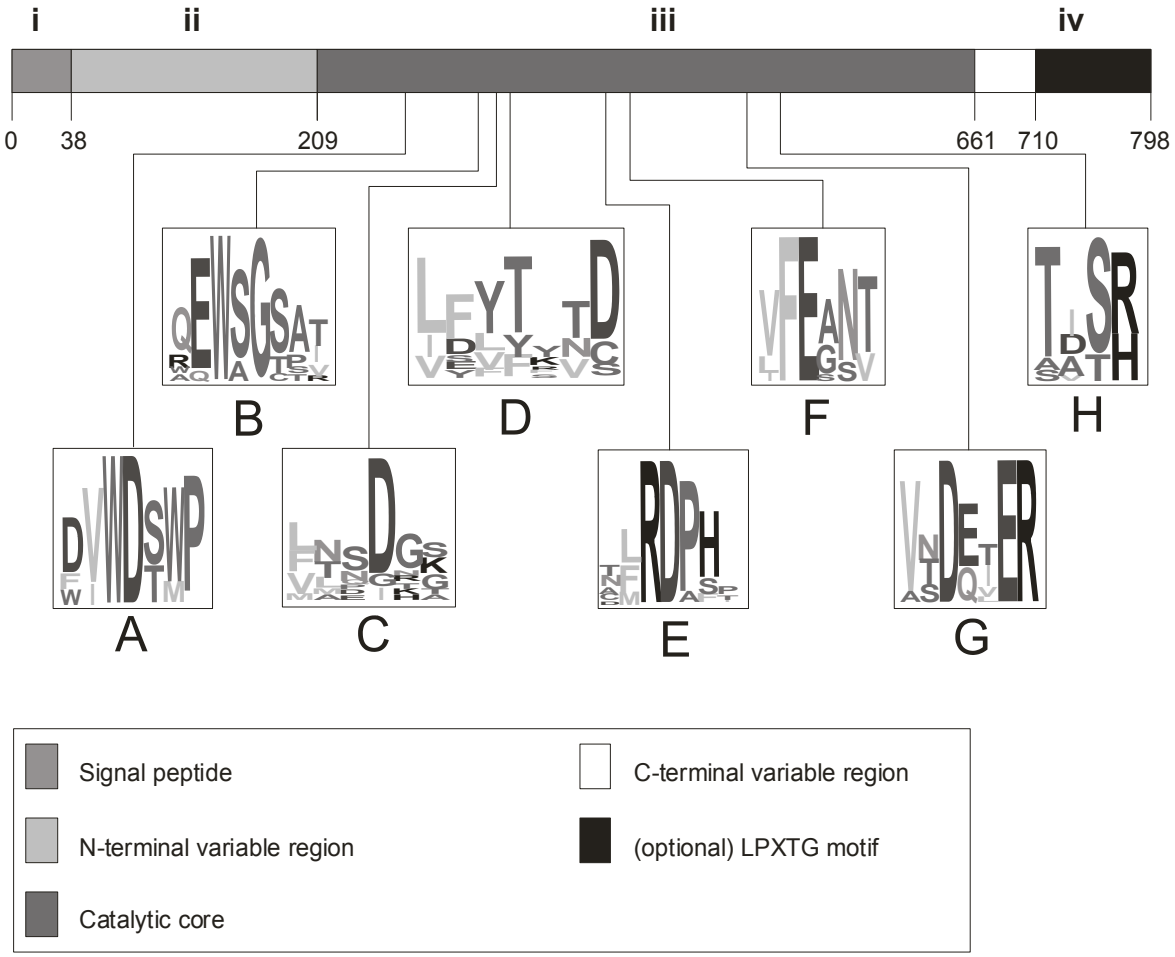

Figure 5. Schematic representation of FTFs (from LAB). The *Lb. reuteri* inulosucrase (Inu) deduced amino acid sequence was used as template (AF459437). The four different regions shown are (i) N-terminal signal sequence; (ii) N-terminal variable region; (iii) catalytic core; (iv) C-terminal variable region (which in some cases contains an LPXTG cell-wall anchor). Alignments (SequenceLogo, http://www.bio.cam.ac.uk /seqlogo/) are shown of short regions in FTF enzymes with conserved amino acid residues for which mutant information is available in literature. Representative members from several groups of FTF enzymes (see Fig. 8) were used for these alignments: *B. subtilis* SacB, *Geobacillus stearothermophilus* SacB, *B. amyloliquefaciens* SacB, *S. mutans* SacB, *Lb. reuteri* Inu, *Lb. reuteri* Lev, *S. salivarius* SacB, *E. amylovora* SacB, *P. syringae* SacB, *Z. mobilis* SacB, *G. diazothrophicus* SacB. **A** corresponds to Asp86 in *B. subtilis* SacB which is presumably the nucleophile based on a crystal structure of an inactive sucrose-bound mutant Glu342Ala of the same enzyme [106] and biochemical characterization of the Asp272Asn mutant of *Lb. reuteri* Inu [123]. **B** corresponds to Glu117Gln in *Z. mobilis* SacB which resulted in a higher transglycosylation activity [200]; SGSA, "sucrose binding box 1", a conserved region between sucrose utilizing enzymes [145]. **C** corresponds to Asp312Ser in *S. salivarius* FTF (possibly involved in acception recognition and / or stabilizing a beta turn in the protein) [154]. **D** corresponds to "sucrose binding box 2", a conserved region between sucrose utilizing enzymes [145]. **E** corresponds to the RDP motif; Asp397Ser in *S. salivarius* FTF (sucrose hydrolysis and polymerization ceased) [154], Asp309Asn in *G. diazotrophicus* LsdA (catalytic activity reduced 75 fold) [12], Asp194Asn in *Z. mobilis* SacB (3.400 fold decrease in catalytic activity) [200], and Asp247 in *B. subtilis* SacB and Asp272 in *Lb. reuteri* Inu (stabilizer of the oxocarbenium ion-like transition state) [106,123]. **F** corresponds to Glu211Gln in *Z. mobilis* SacB (28% of retained sucrose hydrolysis, but highly reduced transfructosylation). **G** corresponds to Glu278Asp (30 fold lower catalytic activity), Glu278His (virtually inactive enzyme) in *Z. mobilis* levansucrase SacB [200], and Glu342 in *B. subtilis* SacB and Glu523 in *Lb. reuteri* Inu (general acid) [106,123]. **H** corresponds to Arg331His (higher oligo formation) in levansucrase from *B. subtilis* SacB [25].

7.2. The core region

The tertiary structure of the *B. subtilis* levansucrase has provided important insights in functional roles of several conserved amino acid residues in this core region [106]. Firstly, the region conserved between all GH68 family members (Fig. 5A) contains an Asp residue which was identified as a nucleophile based on the 3D structure analysis of *B. subtilis* levansucrase and by the mutagenesis study of its equivalent in *Lb. reuteri* FTFs mutants [106,123](see also Chapter 2). Two further regions that are highly conserved among FTFs and invertases have been designated as "sucrose binding boxes" (SBBs) (Fig. 5B and D) [145]. Yanase *et al.* have presented evidence for direct involvement of SBB1 in catalysis of transfructosylation. In the Glu117Gln mutant (SBB1) of *Z. mobilis* levansucrase the catalytic turnover was similar to that of the wild-type enzyme, while greater transfructosylating activity was observed [200]. Based on the analysis of the activity of site directed mutants Asp312Glu, Asp312Ser, Asp312Asn, Asp312Ile, and Asp312Lys located between SBB1 and SBB2 (Fig. 5C) of *S. salivarius* levansucrase, it has been suggested that this Asp residue is most likely involved in determining acceptor recognition or stabilizing of a β-turn in the protein [154]. Analysis of the 3D structure of SacB revealed that Asp312 indeed forms 180° reverse β-turn between SBB1 and SBB2 and is located on the surface of the protein, far from active site.

An Arg / Asp / Pro amino acid motif (RDP; Fig. 5E) is present in a conserved position in the core region of FTF enzymes, invertases, levanases, inulinases, and sucrose-6-phosphate hydrolases. It is likely to have a common functional role in these enzymes, and this has been studied in more detail for the *S. salivarius*, *G. diazotrophicus*, and *Z. mobilis* levansucrases. Mutation of the Asp amino acid residue in the RDP motif of the latter three enzymes resulted in dramatic decreases of catalytic activities while the affinities for their substrate sucrose remained almost unaltered [12,154,200]. Analysis of the 3D structure of *B. subtilis* SacB revealed that this Asp247 residue in the RDP motif, although required for catalysis, apparently is not directly involved in the FTF reaction mechanism. It is involved in stabilization of the transition state [106].

Catalytic activity of the levansucrase from *Z. mobilis* became reduced dramatically upon mutation of Glu278 (Fig. 5G). Analysis of the sucrose-bound structure of a *B. subtilis* levansucrase inactive mutant (Glu342Ala) revealed that the Glu342 residue, corresponding to the *Z. mobilis* Glu278 residue (Fig. 5G), functions as a general acid.

Mutation of Arg331 in the *B. subtilis* levansucrase (Fig. 5H) yielded enzymes that were differently affected in transfructosylation activity dependent on the substitution chosen; three variants (Arg331Lys, Arg331Ser, and Arg331Leu) had lost the ability to synthesize levan and were only able to produce the trisaccharide kestose (GFF). It has been suggested that the side chain of Arg331 could act as a proton donor in the bifunctional catalysis [25]. Analysis of the three-dimensional structure of the *B. subtilis* levansucrase showed, however, that this Arg331 residue is structurally involved in the acceptor binding site [106]. Similar conclusions were drawn from an experiment where the Arg331 counterpart in *Z. mobilis* levansucrase (His296) was mutagenized.

Replacement of this residue by Arg led to the reduction of transfructosylation activity and accumulation of small amount of oligosaccharides [200].

Evidence concerning the role of the core region in determining enzyme activity was obtained from the characterization of the inulosucrase gene from *Ln. citreum* CW28 (Table 3). This enzyme has the structural arrangement of a GTF enzyme, namely alternansucrase from *Ln. mesenteroides* NRRL B-1355, in which the catalytic domain is substituted by that of an FTF enzyme. Its N-terminal region is similar to the variable region of GTF enzymes, its catalytic domain is similar to the core region of FTFs from various bacteria, and its C-terminal domain presents similarity to the glucan binding domain (GBD) from alternansucrase [120]. Truncations of the C-terminal domain yielded an active inulosucrase enzyme that had lost thermal stability [120].

A most interesting question is what structural features in FTF enzymes determine their specificity for synthesis of either β(2-6) or β(2-1) linkages, and the sizes of fructans produced. Recently published comparison of 3D structure of *G. diazotrophicus* levansucrase and *B. subtilis* levansucrase revealed, that enzymes synthesizing FOS or large polymer posses identical active site [104]. Lack of structural data for complexes between fructosyltransferases and fructosyl acceptors makes it difficult to understand what determines polymer vs. short oligosaccharides synthesis ratio.

7.3. The C-terminal domain

The function of the C-terminal region of FTFs has not been studied extensively. Suggestions for a possible function for this region has been obtained in studies with the levansucrase gene of *B. subtilis* [29]. Readthrough of a stop-codon enabled a downstream region to enlarge the gene at the 3'-end. Site-directed mutagenesis of this stop codon yielded a mutant *B. subtilis* levansucrase protein that had become enlarged by 3 kDa. This resulted in an enzyme that synthesized a larger levan polymer than the wild-type levansucrase. The increase in levan molecular weight was caused mainly by an increase in the number of branches in the levan. This implies that this C-terminal extension affects one way or the other the degree of branching of the synthesized polymer. Moreover, the extended enzyme was able to form an active dimer from two polypeptide chains linked by an S-S bridge [29].

A variety of proteins from Gram-positive organisms such as *Staphylococcus aureus* [105], *Listeria monocytogenes* [19], and *Streptococcus pyogenes* [11] (including some bacterial FTF enzymes, see below), carry a C-terminal LPXTG motif which is responsible for cell-wall anchoring. The LPXTG motif cell-wall anchor consists of (i) a spacer region (of 50 to 125 amino acid residues) rich of Pro/Gly and / or Thr/Ser residues [49], (ii) the well-conserved sorting signal LPXTG [49], (iii) a stretch of hydrophobic amino acids (about 30) [115], and (iv) two to three positively charged amino acids (Lys, Arg, and His). The stretch of hydrophobic residues acts as a membrane-spanning region with the positively charged amino acid residues directed towards the cytosol. The protein is located outside the cytoplasmic membrane with an N-terminal signal sequence attached to the membrane. After proteolytic cleavage of the signal sequence by a signal peptidase

(SPase) the remaining N-terminal part of the protein, spaced by the Pro/Gly and / or Thr/Ser rich region, is directed outwards of the cell. Subsequently, the LPXTG motif is proteolytically cleaved between the Thr and the Gly residues by a sortase enzyme and covalently linked to the peptidoglycan layer [114,115].

There are a few examples of FTF enzymes that are (possibly) cell-wall associated, namely *Lb. reuteri* levansucrase (and inulosucrase) [184,186], *S. salivarius* levansucrase [133], and the periplasmic *Pseudomonas syringae* (non-LAB) levansucrase [97].

A common C-terminal LPXTG cell-wall anchoring motif (Fig. 5) is found in both inulosucrase [189] and levansucrase from *Lb. reuteri* [187]. Although inulo-oligosaccharides were detected in supernatants of *Lb. reuteri* cultures grown in sucrose containing medium, neither the presence of inulosucrase in culture supernatants nor actual cell-wall association of the inulosucrase have been demonstrated [189]. The cell-wall associated *S. salivarius* FTF protein contains a C-terminal region that resembles the LPXTG cell-wall anchoring motif but lacks the actual LPXTG amino acids [133].

Proteins displayed on the bacterial surface may have various functions for the bacterial cell. For the pathogens *S. aureus* and *Enterococcus faecium*, surface proteins are thought to play a major role in the infection process in humans [177]. Surface proteins from urogenital *Lactobacillus* spp. mediate the adhesion with tissue cells [71,150] and play a role in the maintenance of a healthy urogenital microflora. Cell associated EPS, produced by FTF enzymes that are anchored to the cell-surface, may also be involved in adherence of the organism to a surface, such as the intestinal mucosa.

8. Secretion, folding, and thermal stability of FTF proteins

A two-step secretion mechanism has been postulated for the *S. salivarius* levansucrase protein [178]. A similar translocation mechanism has been proposed for the *B. subtilis* levansucrase where two different transient forms of the protein, of 53,000-Mr and 50,000-Mr, were found to be located in the membrane compartment of *B. subtilis* QB112 induced for synthesis of the extracellular form of the levansucrase [128]. The two-step secretion mechanism occurs according to the following sequence: (i) translation of the levansucrase mRNA into a 53,000-Mr protein which is attached to the cellular membrane, (ii) covalent modification yields a protein of 50,000-Mr, and (iii) secretion of the 50,000-Mr form. Processing of the 53,000 M_r to the 50,000 M_r membrane-form of the protein is faster than the conversion of the latter to the extracellular form. Pulse-labelling experiments indicated, that the second step of levansucrase translocation is an active transport step directly linked to the entrance flux of iron into the cell [28]. Under cytosolic conditions (pH 7-7.5, 37°C and low concentrations of Ca^{2+} and Fe^{3+}) the newly synthesized protein remains unfolded. After spontaneous insertion into the membrane bilayer and cleavage of the signal sequence, the protein is released extracellularly and refolded. This event is catalyzed by metal ions such as Ca^{2+} and Fe^{3+} [130]. The presence of one, well-defined calcium binding site in *B. subtilis* levansucrase and the importance of

Ca^{2+} and Fe^{3+} ions for the efficiency of secretion and the folding process of the enzyme have been demonstrated previously [129,130].

Expression of FTF enzymes of Gram-positive bacteria is in most cases induced by sucrose, e.g. *B. subtilis* levansucrase [126], *S. salivarius* FTF [107], and *Lb. reuteri* levansucrase [51]. This could be due to increased de novo synthesis and / or caused by release of the enzyme from the cell-wall [186]. Lee *et al.* hypothesize that *S. mutans* senses the sucrose level in the environment, resulting in derepression of *ftf* transcription at low sucrose concentrations [96]. Induction of FTF enzyme expression may also be independent of the carbohydrate source, e.g. in case of *A. naeslundii* levansucrase [14]. Growth phase-dependent regulation of levansucrase expression has been reported for the Gram-negative bacterium *R. aquatilis* [147]. Milward *et al.* reported that the activity of *S. salivarius* FTF enzyme is dependent on the presence of Ca^{2+} and this dependence follows standard Michaelis-Menten kinetics consistent with Ca^{2+} being a cofactor for enzyme activity [74,107]. Analysis of the role of calcium ions in fructosyltransferases of *Lb. reuteri* has been published recently by Ozimek *et al.* [122] (see also Chapter 3). In the presence of Ca^{2+} Inu and Lev showed considerably enhanced acivities at high temperatures and thermostability. The activity and stability of FTF enzymes of Gram-negative origin, such as levansucrase of *R. aquatilis* and levansucrase of *P. syringae*, apparently are not affected by the presence of calcium [67,119].

Analysis of the 3D structure of *B. subtilis* levansucrase has provided evidence for a presence of a bound metal ion, most likely Ca^{2+} [106]. Asp339 of *B. subtilis* levansucrase was identified as one of the residues coordinating Ca^{2+} ion in enzyme structure. The functional role of this residue has been studied by mutating equivalent amino-acid residues in Inu and Lev from *Lb. reuteri*, Asp520Asn and Asp500Asn, respectively [122]. Both mutants showed decreased optimal temperature and apparent affinity for Ca^{2+} binding was reduced significantly 1600-fold and 35-fold, respectively. Sequence alignment of family GH68 members revealed that residues involved in binding of the calcium are conserved in most enzymes from Gram-positive bacteria and are absent in proteins of Gram-negative origin [122]. Most likely the disulphide bridge found in the 3D structure of *G. diazotrophicus*, a member of the later group, plays a role similar to calcium ions in enzymes from Gram-positive bacteria [104].

For the levansucrase enzyme of *B. subtilis*, it has been shown that Gly387 residue present in the core region was involved in the metal-dependent refolding of the protein after secretion by the organism [130].

9. Putative FTF enzymes identified in Gram-positive bacteria

As shown in Table 3, several additional (but so far putative) FTF enzymes have been identified in *Arthrobacter*, *Bacillus*, *Clostridium*, *Streptococcus*, *Leuconostoc*, and *Lactobacillus*. Although their sizes are quite divergent, the core regions are all around 450 amino acids. Since the characteristic residues of the sucrose binding boxes and the

catalytic triad are present, these enzymes may very well be active FTF enzymes. The putative *Lactobacillus johnsonii* and *Lactobacillus gasserii* FTF genes (Table 3) cluster closely to the *Lb. reuteri* inulosucrase, which might indicate their inulosucrase identity.

10. Scope of the thesis

This thesis focuses on the structure/function relationships of fructosyltransferase enzymes from *Lb. reuteri* 121. Most LAB synthesizing fructans produce a levan, composed of β(2-6)-linked fructose units, and only a few LAB synthesize a β(2-1)-linked fructan polymer – an inulin. *Lb. reuteri* 121 possesses an inulosucrase (Inu) synthesizing inulin and a levansucrase (Lev) catalyzing levan synthesis. These two enzymes hydrolyze sucrose releasing monosugars or synthesizing fructan polymers and fructooligosaccharides. Inu and Lev are very similar at the amino acid sequence level but differ in the type of polymer synthesized and in the FOS vs. polymer synthesis ratio. The aims of the research described in this thesis were (i) identification of the catalytic triad of the two *Lb. reuteri* 121 FTF enzymes, (ii) identification of the residues involved in calcium binding, and determination of the effects of calcium ions on FTF thermostability, (iii) detailed characterization of substrate specificity and analysis of the oligosaccharide and polymer products synthesized by Inu and Lev, and (iv) identification of amino acid residues involved in determination of the size of products synthesized by Inu.

Chapter 1 reviews current knowledge about fructosyltransferases, their structure and activities.

Chapter 2 describes the identification of the catalytic triad in Inu and Lev. Single mutants of the three catalytic residues were constructed and characterized biochemically. Additionally, changes in mutant enzyme structures were analyzed.

In **Chapter 3,** we analyzed the influence of calcium ions on activity and thermostability of both FTFs. Also, the apparent affinity of Inu and Lev for binding of Ca^{2+} ions and the location of the calcium-binding sites in both enzymes were identified.

Chapter 4 reports a detailed characterization of the transglycosylation reaction products synthesized by Inu and Lev. Inu mainly synthesized a broad range of FOS, whereas Lev synthesized mainly a large polymer. Both enzymes were also able to utilize raffinose and sucrose-related inulin-type of oligosaccharides as substrates and performed a disproportionation type of reaction. In this chapter, we also propose a model for organization of sugar binding subsites in FTFs from *Lb. reuteri.*

Chapter 5 describes a biochemical characterization of three site-directed mutants of Inu. All three mutated residues were located in the active site of the enzyme. One mutation caused virtual inactivation of the enzyme while two other mutations caused clear changes in the ratio of polymer vs. FOS synthesis and the overall spectrum of the oligosaccharides produced. The data suggest that residues located at the –1 subsite are important in determinating of the size of the transglycosylation products.

Table 3. Characteristics and sequence motifs of (putative) FTF enzymes of (non-) LAB (only gene sequence information available). As reference, the *Lb. reuteri* Inu and Lev are used. The nucleophile and acid/base catalysts have been identified in *B. subtilis* SacB and *Lb. reuteri* Lev and Inu proteins [106]. Residues or motifs indicated in boldface (top row): the RDP motif (Fig. 5E), initially identified in the *G. diazotrophicus* levansucrase [12], E211 residue, identified in the *Z. mobilis* levansucrase [200], and R331 residue, identified in the *B. subtilis* levansucrase [25]. Sucrose boxes are further discussed in Fig. 10. * identified in a sequenced genome.

Organism	Accession number	Size (amino acids)	Nucleophile	sucrose box 1	sucrose box 2	RDP	E211 *Z. mobilis*	Acid/base catalyst	R331 *B. subtilis*	Position and size of core region
Arthrobacter globiformis IFO 3062 putative levansucrase	AB125971	548	D300	QWSGS	LFFTDVA	F**RDP**F	VF**E**GNS	VT**D**QTER	SH**R**ST	62-534
Bacillus licheniformis putative levansucrase	AX954581	482	+	EWSGS	LFYTAFS	MRDPH	VFEANT	VTDEIER	SRGSK	20-481
Streptococcus mutans UA159 putative inulosucrase*	AE015025	795	+	EWSGS	LFYTKVD	LRDPH	VFEAST	VSDELER	SRLNH	173-636
Lactobacillus johnsonii NCC533 putative inulosucrase*	RLJO00913	797	+	QWSGS	LYYTKVD	MRDAH	VFEAST	VSDEIER	TRLNR	200-663
Lactobacillus gasseri ATCC-33323 putative inulosucrase*	RLGA00517	630	+	QWSGS	LYYTKVD	MRDAH	VFEAST	VSDEIER	TRLNR	173-630
Leuconostoc mesenteroides ATCC-8293 putative levansucrase*	RLME01775	1015	+	QWSGS	LYYTKVD	LRDPH	AFEANT	ITDEIER	TRLSK	171-622
Leuconostoc mesenteroides ATCC-8293 putative levansucrase*	RLME01776	782	+	QWSGS	LFYTKTD	LRDPH	TFESNT	ITDEIER	TRLSK	166-618
Leuconostoc mesenteroides ATCC-8293 putative levansucrase*	RLME01780	1002	+	QWSGS	LFYTQVD	LRDPH	TFEGST	VTDEIER	ARLDR	167-611
Clostridium acetobutylicum ATCC-824D putative levansucrase*	RCA03451	489	+	EWSGS	LFYTDFS	LRDPH	VFEANT	VTDEIER	SRGSK	24-485
Lactobacillus reuteri 121 Inu E.C. 2.4.1.9	AAN05575	798	+	EWSGS	LFYTRVD	MRDAH	VFEAST	VSDEIER	TRLNR	200-662
Lactobacillus reuteri 121 Lev E.C. 2.4.1.10	AAO14618	804	+	EWSGS	LFFTSND	LRDPH	VFEANT	ASDEVER	TRVSR	177-642

Chapter 2

Site-directed mutagenesis study of the three catalytic residues of the fructosyltransferases of *Lactobacillus reuteri* 121

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Abstract

Bacterial fructosyltransferases (FTFs) are retaining-type glycosidases that belong to the family 68 of glycoside hydrolases (GH68). Recently, a high-resolution 3D structure of the *Bacillus subtilis* levansucrase has been solved [Meng, G. and Futterer, K., 2003, Nat. Struct. Biol. 10, 935-941]. Based on this structure, the catalytic nucleophile, general acid/base catalyst, and transition state stabilizer were identified. However, a detailed characterization of site-directed mutants of the catalytic nucleophile has not been presented for any FTF enzyme. We have constructed site-directed mutants of the three putative catalytic residues of the *Lactobacillus reuteri* 121 levansucrase and inulosucrase and characterized the mutant proteins. Changing the putative catalytic nucleophiles D272 (inulosucrase) and D249 (levansucrase) into their amido counterparts resulted in a 1.5-$4 \cdot 10^5$ times reduction of total sucrase activity.

1. Introduction

Fructosyltransferase (FTF) enzymes belong to the glycoside hydrolase family 68 (GH68) [65] and synthesize either inulin, composed of β(2-1) linked fructose residues (inulosucrase, Inu; EC 2.4.1.9) or levan, composed of β(2-6) linked fructose residues (levansucrase, Lev; EC 2.4.1.10) [186,188]. They are β-retaining enzymes employing a double displacement mechanism that involves the formation and hydrolysis of a covalent glycosyl-enzyme intermediate, with an oxocarbenium ion-like transition state [88]. The active site of members of the GH68 family is composed of a catalytic triad: a catalytic nucleophile, a general acid/base catalyst, and a transition state stabilizer. The catalytic nucleophile attacks the anomeric centre of the sugar, generating the enzyme-substrate intermediate. In a subsequent step this intermediate undergoes either transglycosylation or hydrolysis. Both steps require assistance of a general acid/base catalyst and a transition state stabilizer.

Very recently, the 3D structure of the *B. subtilis* levansucrase with sucrose bound in the active site was resolved [106], the first of the GH68 family. The crystallographic data showed that D86 and E342 were in the proper position and in close contact to the sucrose to represent the catalytic nucleophile and general acid/base catalyst, respectively. Alignments showed that residue E342 is equivalent to the invariant Glu of yeast invertase and *Zymomonas mobilis* levansucrase (E278), which are vital to catalysis according to site-directed mutagenesis studies [200]. Residue D247 was identified as transition state stabilizer based on the observation that it forms strong hydrogen bonding with the C3' and C4' hydroxyls of the fructosyl unit, but it is too far away from either the C2' hydroxyl or the glycosidic oxygen to be one of the residues directly involved in catalysis. Additional evidence for the role of D247 comes from mutational studies on the equivalent residue in the levansucrases of *Zymomonas mobilis* (D194), *Streptococcus salivarius* (D397) and *Gluconacetobacter diazotrophicus* SRT4 (D309) [12,154,200]. With respect to the catalytic nucleophile, no experimental mutagenesis studies have been reported yet.

Lactobacillus reuteri 121 possesses two fructosyltransferase (*ftf*) genes encoding an inulosucrase (Inu; GenBank accession number AF459437) and a levansucrase (Lev; GenBank accession number AF459437). We characterized site-directed mutants of the catalytic triad in the two *Lb. reuteri* FTFs. This is the first report on mutagenesis data of the catalytic nucleophile of levansucrases and inulosucrases, showing that when this residue is changed into its amido counterpart the sucrase activity is lost completely.

2. Materials and methods

2.1 General

Plasmid pBAD/myc/his/C (Invitrogen) was used for cloning purposes. *Escherichia coli* strain Top10 (Invitrogen) was used for gene expression. Plasmid-carrying *E. coli* strains were grown at 37°C on LB medium [142] with 100 µg ml^{-1} ampicillin and 0.02 % (w/v) arabinose. Proteins were produced with a C-terminal truncation of 32 amino acid residues and a C-terminal (poly)-Histidine tag, and purified, as described [189]. Protein concentrations were determined using the Bradford reagent (Bio-Rad, Munich, Germany) with bovine serum albumin as standard.

2.2 Site-directed mutagenesis

Single mutations were introduced using the 'megaprimer' method [143] and confirmed by sequencing. *Pwo* polymerase (Roche biochemicals) was used for all PCR reactions using plasmid pBAD containing the *Lb. reuteri* 121 *lev* or *inu* genes as templates. PCR products were digested by *Nco*I and *Bgl*II and ligated into the pBAD vector, downstream of an inducible arabinose promoter and upstream of a His tag. For site-directed mutagenesis (synthesis of 'megaprimers') the following oligonucleotides were used in PCR reactions:
Ad272n-I, 5`-GCCATGAATTCCATACATCTAAAGG-3` (Inu D272N); Ad424n, 5`-CGATAATATAGCAATGCGTAATGCTCATG-3` (Inu D424N); Ae523q, 5`-GCGATGAAATTCAGCGGCCGAATGTAG-3` (Inu E523Q); Bd249n-I, 5`-GTACGGGCCAAGAGTTCCAGACATC-3` (Lev D249N); Bd404n, 5`-CTGCTTACGTAACCCACACGTTGTTC-3` (Lev D404N); Be503q, 5`-GTGATGAAGTTCAACGGCCGAACGTAG-3` (Lev E503Q).
Additionally, two flanking primers were used in all reactions: pBADRV-I, 5`-TCTGAGATGAGTTTTTGTTCGG-3`; pBADFD, 5`-TCCTACCTGACGCTTTTTATCG-3`. The underlined codons indicate mutations introduced; -I: antisense primer.

2.3 Activity measurements

Total activity of purified enzymes was determined by measuring the amount of glucose released from sucrose [186]. The amount of glucose formed reflects the total amount of sucrose utilized during the reaction (V_G). Assays were performed at 37°C in 25 mM sodium acetate buffer pH 5.4 and 1 mM $CaCl_2$ with 250 mM (Inu) or 100 mM (Lev) sucrose, respectively. For the wild-type enzyme samples were taken every 3 min. Because Inu and Lev mutants had low residual activity the assay conditions were modified as

follows: measurements were done with 200 mM sucrose as substrate over a period of several hours with samples taken every 60 min (modified conditions). Although the FTFs are optimally active at 50°C [188], activity measurements over longer time periods were performed at 37°C to aviod abiotic hydrolysis of sucrose. All activity measurements showed linear increases in time and were proportional to the amount of enzyme added. The SigmaPlot 2001 version 7.0 was used for curve fitting of the data with the standard Michaelis-Menten formula. The kinetic parameters were determined at 37°C using sucrose concentrations ranging from 1 mM to 1 M. Enzyme activity assays were performed in triplicate.

2.4 Circular dichromism (CD) spectra

Multiple far-UV CD spectra (195 – 260 nm) were recorded at pH 7 and 37°C on a Jasco J-715 spectropolarimeter (Jasco, Japan) in quartz cells (optical path length of 1 mm); scan speed 100 nm/min; data interval 0.2 nm; band width 1.0 nm; sensitivity 20 mdeg; response time 0.125 s. All recorded spectra were corrected by subtraction of the spectrum of a protein free sample. Analysis of the spectra for their secondary structure content was performed according to [38]. Multiple near-UV CD spectra (250-350 nm) were recorded at pH 7 and 37°C using a cell with an optical path length of 1.0 cm; scan speed 50 nm/min; data interval 0.5 nm; band width 1.0 nm; sensitivity 10 mdeg; response time 0.25 sec. All recorded spectra were corrected by subtracting spectrum of protein free sample.

3. Results and discussion

3.1 Site-directed mutagenesis of the catalytic triad

Very recently, the 3D structure of the *B. subtilis* levansucrase was published [106]. The residues D86, D247, and E342 were identified as respectively the catalytic nucleophile, the transition state stabilizer and the general acid/base catalyst. Mutations in these residues, introducing alanine residues at these positions, resulted in loss of activity. The enzyme activity assay used measured levan production by monitoring changes in optical density in time of a reaction mixture with buffer, sucrose and enzyme. Earlier studies have reported mutagenesis data for the putative general acid/base catalyst or the transition state stabilizer in FTF enzymes, but the individual mutations were made in different enzymes. No mutagenesis data on the catalytic nucleophile of FTF enzymes have been reported. Thus, a comparative site-directed mutagenesis study combined with a determination of the resulting catalytic activity and the kinetic properties for one and the same enzyme has not been published.

```
SACB_BACSU     82  LDVWDSWPLQ     244  TLRDPHY     337  VTDEIERA
INU_LACRE     268  LDVWDSWPVQ     421  AMRDAHV     518  VSDEIERP
LEV_LACRE     245  LDVWDSWPVQ     401  CLRDPHV     498  ASDEVERP
                   ********:*          :**.*          ..*: **.
```

Figure 1. Multiple sequence alignment (created using Clustal X (1.5b) program) [172] of bacterial FTF proteins, revealing three conserved blocks. The first conserved block contains the so-called VWDS motif and the second conserved block contains the so-called RDP motif. Asterics indicates fully conserved residues. Residues subjected to mutagenesis are shown in bold. SACB_BACSU – *B. subtilis* SacB; INU_LACRE – *Lb. reuteri* inulosucrase; LEV_LACRE – *Lb. reuteri* levansucrase.

Based on a sequence alignment of *Lb. reuteri* 121 Lev and Inu and the *B. subtilis* levansucrase (Fig. 1), residues D249, D404 and E503 (Lev) and D272, D424, and E523 (Inu) were identified as the putative catalytic nucleophile, the transition state stabiliser and the general acid/base catalyst, respectively. These residues were changed into their amido counterparts by site-directed mutagenesis and the total activity, i.e. the release of glucose from sucrose was measured. The activity of the mutants was reduced at least 10,000-fold compared to the wild-type enzymes (Table 1). This is the first report on mutagenesis of the catalytic nucleophile in FTF enzymes, showing that when this residue is changed into its amido counterpart, enzyme activity is lost virtually completely.

Table 1. Total activity (U/mg) of purified *Lb. reuteri* 121 inulosucrase and levansucrase wild-type and site-directed mutant proteins

Inu		**Lev**	
Wild-type	47 ± 4	wild-type	71 ± 15
D272N	$3.2\cdot10^{-4} \pm 2.8\cdot10^{-4\#}$	D249N	$1.8\cdot10^{-4} \pm 10^{-4\#}$
D424N	$6.6\cdot10^{-4} \pm 1.1\cdot10^{-4\#}$	D404N	$2.3\cdot10^{-4} \pm 3.04\cdot10^{-5\#}$
E523Q	$2.8\cdot10^{-3} \pm 1.1\cdot10^{-3\#}$	E503Q	$2.2\cdot10^{-3} \pm 1.4\cdot10^{-3\#}$

remaining activity sampled every 1 hour

Kinetic properties could only be determined for the Lev site-directed mutants D404N and E503Q (Table 2). The k_{cat} for the D404N mutant had decreased 613,000-fold, with an almost unchanged K_m. A considerably decreased k_{cat} value (68,000-fold) also was determined for the E503Q mutant, whereas its K_m decreased about eight-fold (from 21 to 2.6 mM). Unfortunately, the assay sensitivity could not be increased enough to determine kinetic parameters for the four other mutants. The k_{cat} values of these four other mutants are at least 10^5-fold lower compared to the wild-type enzymes (Table 2).

The large reduction in V_G of *Lb. reuteri* 121 Inu mutants (D272N, D424N and E523Q) and Lev mutants (D249N, D404N and E503Q) suggests that these residues are essential for catalysis. The E503Q mutant showed a relatively small reduction in k_{cat}/K_m ratio (Table 2), suggesting that the E503 (Lev) and E523 (Inu) residues act as general

acid/base catalysts, similar to E342 of *B. subtilis* levansucrase [106] (Fig. 1). Our data support the hypothesis [106] that the Asp residue located in the highly conserved family GH68 motif –V**WD**SW- (invariant residues in bold) acts as catalytic nucleophile (Fig. 1): D249 (Lev) and D272 (Inu) residues. Finally, we conclude that D404 (Lev) and D424 (Inu) in the highly conserved family GH68 RDP motif (Fig. 1) constitute the third residue (transition state stabilizer) of the catalytic triad in these *Lb. reuteri* 121 enzymes.

Table 2. Kinetic parameters of purified *Lb. reuteri* 121 levansucrase wild-type and site-directed mutant proteins.

	Kinetic parameters		
	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}sec^{-1}$)
wild-type	184 ± 17	21 ± 4.1	8.7
D249N	n.d.	n.d.	n.d.
D404N	$3 \cdot 10^{-4} \pm 2.1 \cdot 10^{-5}$	28 ± 11	$1.07 \cdot 10^{-5}$
E503Q	$2.7 \cdot 10^{-3} \pm 1.3 \cdot 10^{-3}$	2.6 ± 0.75	$1.0.10^{-3}$

[n.d.] not determined

3.2 Secondary / tertiary structure of inactive Inu and Lev mutants

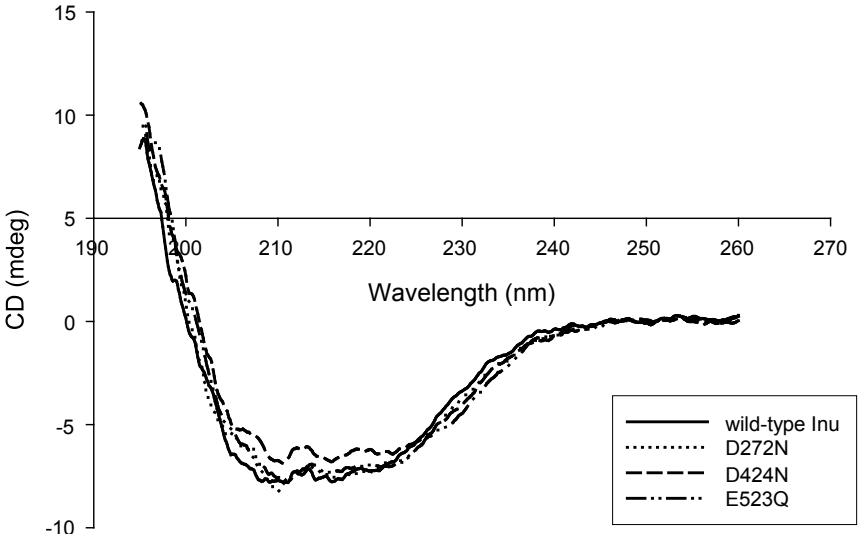
Mutations in the catalytic residues of the *B. subtilis* levansucrase caused minor, if any, structural changes in the protein [106]. To determine whether mutations in the catalytic residues of the *Lb. reuteri* 121 levansucrase and inulosucrase had caused structural changes, we recorded far- and near-UV CD spectra of the wild-type and the mutant enzymes. Compared to wild-type protein especially Lev mutants D424N and E523Q showed slightly more α-helical (Table 3). Near-UV CD spectra of wild-type and mutants of *Lb. reuteri* 121 Inu showed more pronounced differences than the far-UV spectra. The wild-type Inu had a well-defined tertiary structure (Fig. 2). D272N also showed quite strong tertiary interactions, strongly resembling those in the wild-type. The near-UV CD spectra of D424N and D523N were very similar to each other but somewhat different from those of the wild-type and D272N. The tertiary structure of D424N and D523N was probably somewhat more flexible, as indicated by the loss of intensity between 255 and 285 nm. Near-UV CD spectra of Lev mutants showed even smaller differences compared to the wild-type protein, than the Inu mutants. Only the tertiary structure of D249N seemed to have become slightly less flexible compared to wild-type, as indicated by the increase in absolute intensity between 285 nm and 305 nm (Fig. 2). Comparison of the overall structures of these mutants with the wild-type protein revealed, that minor, if any, structural changes had occurred, upon mutations. These data strongly

suggest that the change in the chemical nature of mutated residues is responsible for the inactivation of mutant proteins.

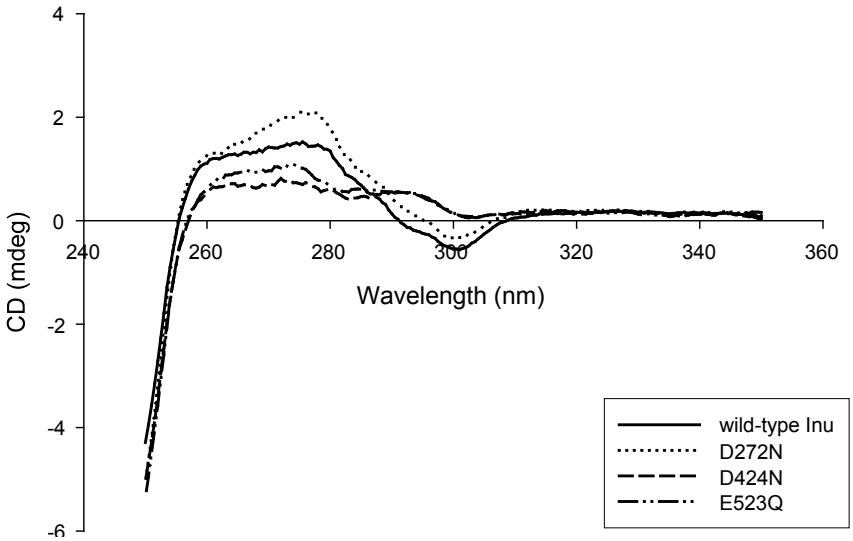
Table 3. Curve fitting estimation of the secondary structure content of *Lb. reuteri* 121 Inu WT and mutant proteins based on analysis of far UV CD spectra.

	α-helix [%]	β-sheet [%]	Unstructered [%]	Turn [%]
Inu				
WT	24	35	35	6
D272N	28	35	33	4
D424N	34	39	27	0
E523Q	34	36	29	1
Lev				
WT	21	25	43	11
D249N	26	23	40	10
D404N	28	18	45	9
E503Q	27	24	42	7

Far-UV



Near-UV



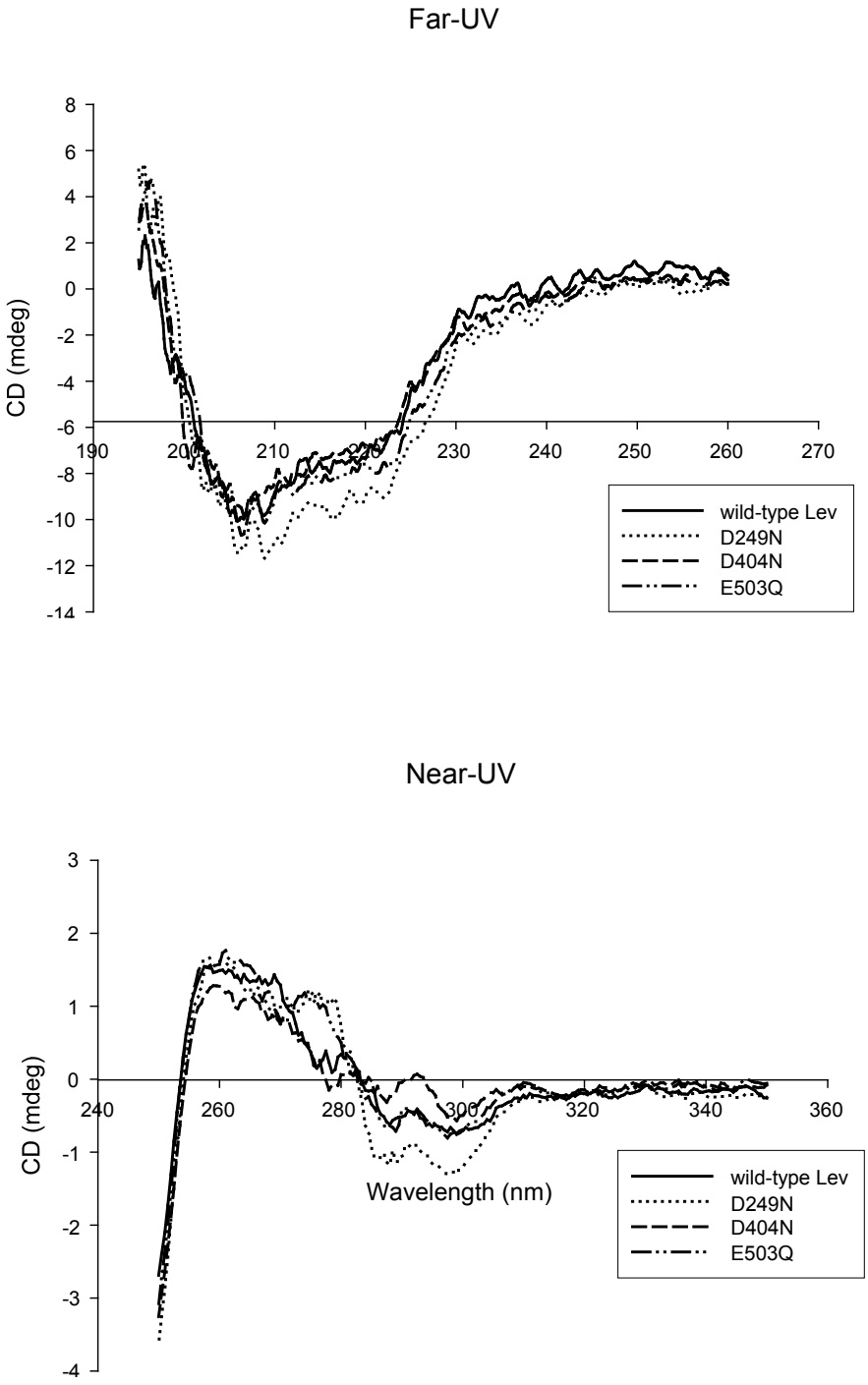


Figure 2. Far-UV (190 – 260 nm) and Near-UV (250 – 350 nm) CD spectra of *Lb. reuteri* 121 Inu and Lev wild-type and mutant proteins recorded at 37°C. Proteins concentrations used: 1.1 – 1.5 mg ml^{-1}.

Chapter 3

Mutational analysis of the role of calcium ions in the *Lactobacillus reuteri* strain 121 fructosyltransferase (levansucrase and inulosucrase) enzymes

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Abstract

Bacterial fructosyltransferase (FTF) enzymes belonging to glycoside hydrolase family 68 (GH68) are not known to require a metal cofactor. Here we show that Ca^{2+} ions play an important structural role in the *Lactobacillus reuteri* 121 levansucrase (Lev) and inulosucrase (Inu) enzymes. Analysis of the *Bacillus subtilis* levansucrase 3D structure [Meng, G. and Futterer, K., Nat. Struct. Biol. 10 (2003) 935-941] has provided evidence for the presence of a bound metal ion, most likely Ca^{2+}. Characterization of site-directed mutants in the putative Ca^{2+} ion-binding sites of *Lb*. *reuteri* Lev and Inu revealed that the Inu Asp520 and Lev Asp500 residues play an important role in Ca^{2+} binding. Sequence alignments of family GH68 proteins showed that this Ca^{2+} ion-binding site is (largely) present only in proteins of Gram-positive origin.

1. Introduction

FTF enzymes are found in Gram-negative and Gram-positive bacteria (see CAZY database: http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html) [34]. They convert sucrose into fructan polysaccharides, in most cases with β(2-6) glycosidic bonds (a levan). A few FTF enzymes of Gram-positive bacteria synthesize inulin, with β(2-1) glycosidic bonds [120,145,189]. Previously, we have characterized the *Lb. reuteri* strain 121 levansucrase (Lev; EC 2.4.1.10) [187] and inulosucrase (Inu; EC 2.4.1.9) [188] enzymes, and identified the catalytic triad in both enzymes [123]. These two FTF enzymes are very closely related (86 % similarity and 56 % identity over 768 amino acids), both depending on Ca^{2+} ions for activity (albeit to a different extent) and displaying unusually high temperature optima (about 50°C) (see Results). They differ most clearly in fructans synthesized, levan and inulin. Fructan synthesis by FTF enzymes of this (probiotic) *Lb. reuteri* strain is of strong interest for food and nutrition applications.

Analysis of the first high-resolution (1.5 Å) 3D structure of a *B. subtilis* levansucrase [106] has provided evidence for the presence of a bound metal ion, most likely Ca^{2+}. Amino acid residues involved in this putative Ca^{2+} ion-binding site are conserved in most of the FTF proteins from Gram-positive bacteria, but not in all members of family GH68 (bacterial FTF and invertase enzymes) [106] (see Chapter 3). In *B. subtilis* levansucrase, Asp339 was suggested to make a most important contribution to Ca^{2+} binding. Data for FTF Asp339 mutant enzymes have not been reported yet. In the present study we have analyzed the (differences in) sensitivity of the *Lb. reuteri* strain 121 Lev and Inu enzymes for Ca^{2+} ions and EDTA. Moreover, the suggested role in Ca^{2+} binding of residues Asp500 (Lev) and Asp520 (Inu), equivalent to Asp339 in *B. subtilis* levansucrase, was probed by site-directed mutagenesis.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Escherichia coli strain Top10 (Invitrogen) was used for expression of wild-type (WT) and mutant *Lb. reuteri* 121 *ftf* genes, inulosucrase (*inu*; GenBank accession number AF459437) and levansucrase (*lev*; GenBank accession number AF465251). Plasmid pBAD/myc/his/C (Invitrogen) was used for cloning purposes. Plasmid carrying *E. coli* strains were grown at 37°C on LB medium [142] supplemented with 100 µg/ml ampicillin and 0.02 % (w/v) arabinose for *ftf* gene induction. WT and mutant proteins were expressed in *E. coli* as constructs with a C-terminal truncation of 32 amino acid residues, and a C-terminal poly-histidine tag [189].

2.2. Molecular techniques

Alignments of FTF and invertase enzymes of family GH68 (CAZY, URL: http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html) [34] were constructed using the Clustal X (1.5b) program. Using site-directed mutagenesis, the *Lb. reuteri* 121 Lev Asp500 and Inu Asp520 amino acids were replaced by Asn and Ala residues. Single mutations were introduced in the *inu* and *lev* genes using the "megaprimer" method [143] and were confirmed by sequencing. PCRs with *Pwo* polymerase (Roche biochemicals) used plasmid pBAD/myc/his/C containing the *lev* or *inu* genes as templates. All PCR products were digested with *Nco*I and *Bgl*II and ligated into the pBAD/myc/his/C vector, downstream of an inducible arabinose promoter and upstream of a His tag. For site-directed mutagenesis (synthesis of "megaprimers") the following oligonucleotides were used in PCRs:
AD520A, 5`-CCAATGGTAAGCGCTGAAATTGAG-3` (Inu D520A); BD500A, 5`-GGCTAGTGCTGAAGTTGAACGAC-3` (Lev D500A); BD500N-I, 5`-CGTTTGGTCGTTCAACTTCATTACTAGCCATC-3` (Lev D500N); AD520N, 5`-CAATGGTAAGCAATGAAATTGAG-3` (Inu D520N).
Additionally, two flanking primers were used in all reactions: pBADRV-I, 5`-TCTGAGATGAGTTTTTGTTCGG-3`; pBADFD, 5`-TCCTACCTGACGCTTTTTATCG-3`. The underlined codons indicate mutations introduced; -I: antisense primer.

2.3. Improved expression of lev

SDS-PAGE analysis of Ni-NTA affinity chromatography purified Lev revealed co-purification of two major protein fractions. An additional start codon was recognized in the sequence of *lev*, located at position 685. This start codon was preceded by an imperfect Shine-Dalgarno (SD) sequence (AAGGAA) located 15 bp upstream. To improve protein expression and purification, a silent mutation was introduced in the SD sequence at position 687 (AA**A**GAA) (*bdri* construct). Analysis of purified Lev expressed from *bdri* showed the presence of only a single protein band (not shown). This improved expression construct was used in all experiments presented in this paper.

2.4. Purification of FTF proteins

All proteins produced were expressed in His tag versions and purified by Ni-NTA affinity chromatography as described [189]. MilliQ water was used in all purification steps to minimize the calcium concentration in protein samples. However, no metal ion chelators were added. Purity was checked by SDS-PAGE. Enzyme concentrations were determined using the Bradford reagent (Bio-Rad, Munich, Germany) with bovine serum albumin as standard.

2.5. FTF enzyme activity assays

Activity variations with temperature (22-57°C) were determined in 25 mM sodium acetate buffer pH 5.4 with 100 mM sucrose (and Ca^{2+} ions as indicated), using purified *Lb. reuteri* 121 enzymes, WT Inu (2.9 μg/ml protein) and Inu mutants D520N, A (7.2 μg/ml and 104 μg/ml, respectively), WT Lev (4.5 μg/ml protein) and Lev mutants D500N, A (13.8 μg/ml and 21.6 μg/ml, respectively). At the highest temperatures (Lev mutants 45-55°C, Inu mutants 50-57°C), 5-fold higher protein concentrations were used. After preincubation of the assay mixture at the assay temperature for 5 min, reactions were started by enzyme addition. Samples were taken every 3 min and used to determine the amount of glucose released from sucrose [186]. The amount of glucose formed reflects the total amount of sucrose utilized during the reaction (V_G). Experiments were performed in duplicate. Effects of EDTA were analysed by determining temperature optima of Inu and Lev enzyme activity using the standard assay but with the reaction buffer containing 1 mM EDTA instead of Ca^{2+} ions.

2.6. FTF affinity for calcium ion binding

Lev and Inu enzyme activities were determined as described above with $CaCl_2$ at concentrations of 0 (calcium-free buffer, prepared with MilliQ water) to 10 mM. The data obtained were used to estimate the Ca^{2+} binding affinity of the proteins. The Sigma Plot (version 8.0) program was used for curve fitting of data, with the standard Michaelis-Menten equation: [$y = (ax) / (b + x)$]. In this formula, y is the specific enzyme activity [U/mg], x is the calcium concentration [mM], a is the V_{max} and b is the apparent K_d (K'_d) [mM of calcium].

2.7. Thermostability of Inu and Lev enzymes

The WT Inu (2.9 μg/ml protein) and WT Lev (4.5 μg/ml protein) enzymes were incubated at a range of temperatures (37-60°C) for 30 min with 1 mM calcium or in calcium-free buffer. The remaining enzyme activities were assayed at 37°C according to the standard procedure (see *FTF enzyme activity assays*). Samples preincubated in the presence and absence of calcium were assayed in buffer containing 1 mM calcium or in calcium-free buffer, respectively.

3. Results and Discussion

3.1. Effects of Ca^{2+} ions and EDTA on Lb. reuteri *121 FTF enzyme activity*

Plots of *Lb. reuteri* 121 Inu and Lev specific activity (initial rates) versus temperature in the presence or absence of Ca^{2+} (or EDTA, data not shown) revealed

interesting similarities and differences between the two proteins (Fig. 1). Without addition of EDTA or Ca^{2+}, the *E. coli* produced and purified Lev enzyme lost activity at temperatures above 40°C and was completely inactive at 50°C, whereas Inu activity decreased above 45°C and was completely inactive at 55°C. Addition of 1 mM EDTA strongly reduced the activities of both enzymes. At room temperature there was no effect of EDTA on Inu activity whereas a 60% reduction in Lev activity occurred. The negative effect of EDTA strongly increased with temperature, reaching 65% (Lev) and 40% (Inu) reduction in activities at 30°C and 37°C, respectively, the temperature optima for activity in the presence of EDTA (data not shown). With additional Ca^{2+} ions (1 mM) present, the Lev and Inu enzymes both showed considerably enhanced activities at higher temperatures, Inu now displaying an optimum at 55°C and Lev at 45°C (Fig. 1). The presence of (extra) Ca^{2+} ions thus appeared essential, especially at higher temperatures, to prevent inactivation of both the Lev and Inu enzymes (Fig. 1), most likely by temperature dependent unfolding (see below). These effects of calcium ions and EDTA on both *Lb. reuteri* FTF proteins are in agreement with previously published observations for *B. subtilis* levansucrase [26,28,129,130] and *Streptococcus salivarius* FTF [107,159,178]. Our observations thus suggest that Ca^{2+} ions play an important structural role in these bacterial FTF enzymes and promote the *Lb. reuteri* Lev and Inu enzyme activity at elevated temperatures. Both *Lb. reuteri* WT FTF enzymes showed a high-affinity for Ca^{2+} binding, with $K`_d$ values of 6.9 μM (Lev at 45°C) and 0.48 μM (Inu at 50°C). By comparison, a somewhat lower affinity for Ca^{2+} binding (K_d of 18 μM) has been reported for the *S. salivarius* levansucrase [154].

Chambert *et al*. have shown previously that proper folding of levansucrase of *B. subtilis* depends on the presence or absence of a metal chelator [28]. Our data show that also the two FTF enzymes of *Lb. reuteri* are affected in a similar way by the presence and absence of calcium ions and the chelator EDTA.

3.2. Effects of Ca^{2+} ions on thermostability of Lb. reuteri *121 FTF enzymes*

The Inu and Lev proteins were incubated at a range of temperatures for 30 min, followed by determination of the remaining activity at 37°C. A drastic loss of Inu activity was observed at temperatures above 45-50°C, the presence of Ca^{2+} ions providing clear protection (Fig. 2A). Rather different profiles were obtained for the Lev protein (Fig. 2B). Following incubation of Lev at temperatures of 50-60°C, 50-80% of activity was recovered at 37°C. Also in this case, the presence of Ca^{2+} ions provided protection. A clear reduction in Lev activity (initial rates) was observed at higher temperatures (Fig. 1), but the Lev protein apparently suffered no irreversible damage and recovered activity upon subsequent incubation at 37°C (Fig. 2B). We speculate that this involved a reversible protein unfolding-refolding process. In contrast, the damage of higher temperatures to the Inu protein is more drastic, with no such strong recovery apparent (compare Figs. 1 and 2A).

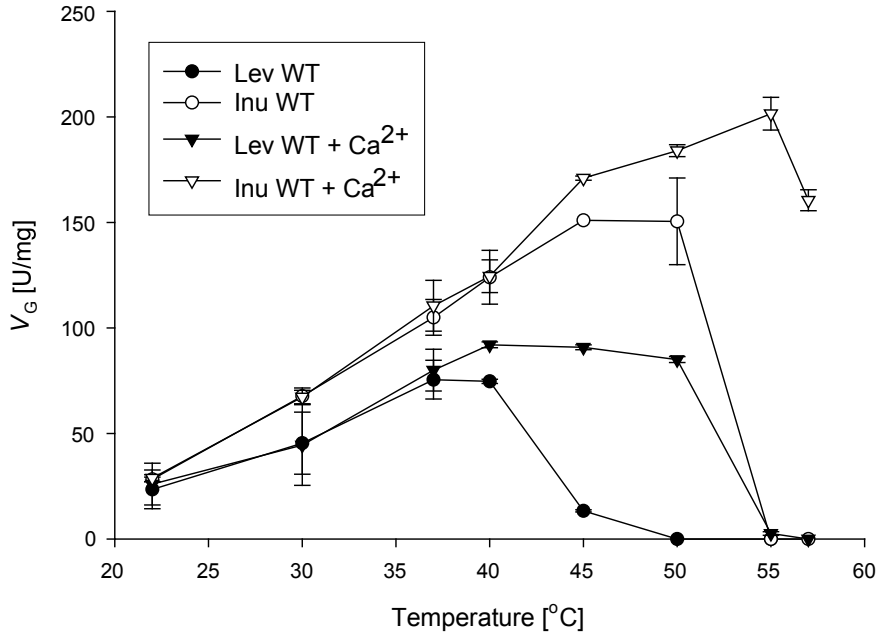


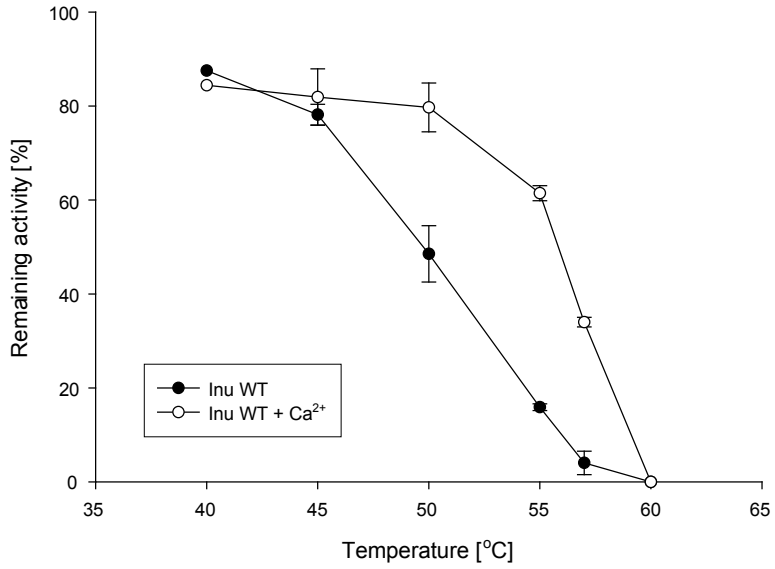
Figure 1. Relationship between the specific activities (V_G) of the *Lb. reuteri* 121 Inu and Lev wild-type enzymes and incubation temperature, measured in the absence or presence of 1 mM Ca^{2+} ions.

3.3. Sequence alignments of members of family GH68

Analysis of the *B. subtilis* levansucrase 3D structure provided evidence for the presence of a metal binding site, most likely for Ca^{2+} ions, showing (in) direct interactions with a number of amino acid residues [106]. This site is penta-bipyramidally coordinated with a water and the carbonyl oxygen of Leu308 on the apices, and with Asp339 Oδ1 and Oδ2, Asn310 Oδ1, Asp241 Oδ1 and Gln272 Oε1 on the equatorial positions. Alignment of all known family GH68 (a total of 22 bacterial FTF and invertase) enzymes revealed that only Asp339 is conserved in all sequences (data not shown). Ca^{2+} ion is coordinated by the *B. subtilis* Asp339 (conserved in all 22 GH68 sequences), present in the conserved DEIER motif, which also contains the general acid catalyst (Glu342 in the *B. subtilis* levansucrase; Glu523 and Glu503 in the *Lb. reuteri* Inu and Lev proteins, respectively) (Fig. 3) [106,123]. Alignment of the 15 known GH68 sequences from Gram-positive bacteria revealed that the 5 residues involved in calcium binding are conserved in most members of this group (Fig. 3). The *B. subtilis* Leu308 is conserved in 8 of these 15 family GH68 proteins of Gram-positive origin, e.g. in *Lb. reuteri* Lev (Leu431), but is present as a Trp, Thr or Tyr residue in the other 7 FTF proteins, e.g. in *Lb. reuteri* Inu (Trp486). The *B. subtilis* Gln272 is conserved in 13 FTF proteins of Gram-positive origin, except for the *Arthrobacter globiformis* beta-fructofuranosidase and the *Leuconostoc citreum* inulosucrase. The *B. subtilis* Asp241 and Asn310 are conserved within all these sequences of Gram-positive origin, except for the *A. globiformis* beta-fructofuranosidase and *Actinomyces naeslundii* FTF. Only Asp339 and none of the other residues forming the putative calcium-binding site is conserved in FTF proteins of Gram-negative origin.

The putative Ca^{2+}-binding site in *B. subtilis* levansucrase thus is (largely) conserved within most of the family GH68 enzymes of Gram-positive origin and is missing in the FTF enzymes from Gram-negative bacteria. Previously, Ohtsuka *et al.* and Hettwer *et al.* have shown that the activity and stability of the levansucrases from the Gram-negative bacteria *Rahnella aquatilis* and *Pseudomonas syringa*e are not affected by the presence of calcium or EDTA [67,119].

A



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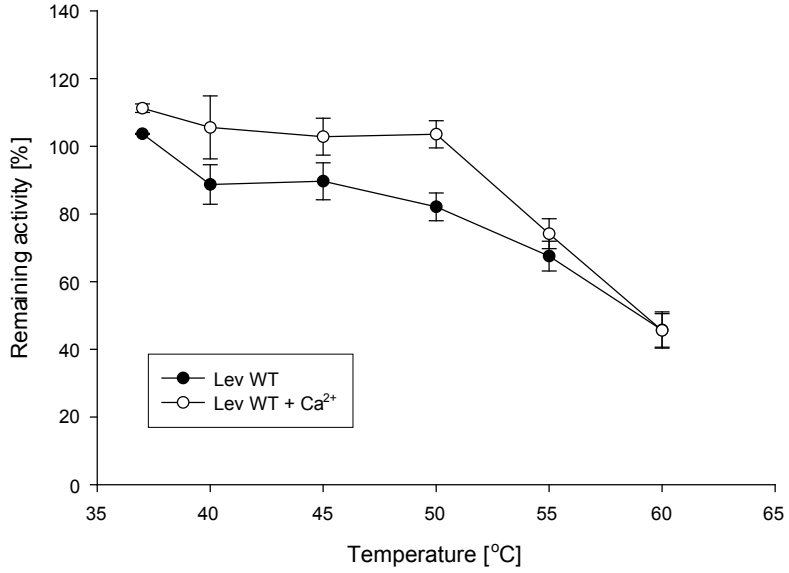

Figure 2. Thermostability of the *Lb. reuteri* 121 Inu (2.9 µg/ml protein) (A) and Lev (4.5 µg/ml protein) (B) wild-type enzymes, measured in the presence and absence of 1 mM Ca^{2+} ions. The relationship between the temperature of pre-incubation (for 30 min) and the remaining specific activity (V_G, measured at 37 °C), is shown.

A low affinity Ca^{2+} binding site (K_d of 1.25 mM, pH 7) has been mapped to Thr265 in the *B. subtilis* levansucrase [130]. This Thr265 hydroxyl hydrogen bonds to Asn310 Nδ2 [106] but is not involved directly in binding of calcium ions. Also the *B. subtilis* Thr265 residue is conserved in 13 out of the 15 GH68 proteins of Gram-positive bacteria, including both the *Lb. reuteri* 121 Inu (Thr443) and Lev (Thr423) (Fig. 3).

SACB_BACSU	238	SSG**D**NHTLRDPH	265	TGTEDGY**Q**GEE	305	TAE**L**A**N**GALG	337	VT**D**EIER
INU_LACRE	415	KGA**D**NIAMRDAH	443	TGLEN-Y**Q**GED	483	RAT**W**A**N**AAIG	518	VS**D**EIER
LEV_LACRE	395	REN**D**DYCLRDPH	423	TGTED-Y**Q**SDD	463	LAG**L**A**N**GALG	498	AS**D**EVER
SACB_STRMU	393	TGA**D**NIAMRDPH	421	TGTEN-Y**Q**GED	461	RAS**W**A**N**AAIG	496	VS**D**ELER
SACB_STRSA	440	DRT**D**NYCLRDPH	473	TGDEN-Y**Q**GEK	507	LAS**W**A**N**GSIG	542	VT**D**EVER
SACB_BACAM	238	TSG**D**NHTLRDPH	265	TGTENGY**Q**GEE	305	DAE**L**A**N**GALG	337	VT**D**EIER
LEV_CLOAC	252	SSG**D**NHTLRDPH	279	TGTNDGY**Q**GDT	320	DAS**L**A**N**GALG	352	VT**D**EIER
LEV_GEOST	238	SSG**D**NHTLRDPH	265	TGTEDGY**Q**GEE	305	TAE**L**A**N**GALG	337	VT**D**EIER
LEV_LACJO	416	KGA**D**NIAMRDAH	444	TGTEN-Y**Q**GDD	484	RAK**W**S**N**AAIG	519	VS**D**EIER
LEV_LACSA	457	RKN**D**DYCLRDPH	485	TGMED-Y**Q**SDD	525	LAS**L**A**N**GAIG	560	AS**D**EVER
LEV_PAEPO	250	VDM**D**NHTFRDPH	277	TGTETGY**Q**GED	317	GAE**L**A**N**GALG	349	VT**D**EIER
UNK_BACLI	247	SSG**D**NHTMRDPH	274	TGTKTGY**Q**GED	314	NAS**L**A**N**GALG	346	VT**D**EIER
FTF_ACTNA	350	----GVNFRDPF	382	SAFVREQ**Q**YVD	430	GGY**Y**Q**L**ANVG	463	VN**D**QTER
BFF_ARTGL	290	----YYNFRDPF	321	SAMDRDE----	354	GAT**Y**Q**I**GNVG	387	VT**D**QTER
INU_LEUCI	492	-QA**D**MFTLRDPK	520	TGIYD-E**A**SDQ	560	YSS**T**A**N**GAIG	595	VT**D**EIER
		: *		:.		. . :*		..*: **

Figure 3. Partial amino acid sequence alignment of family GH68 proteins of Gram-positive bacteria. The 5 FTF amino acid residues forming the strongly conserved putative Ca^{2+} ion-binding site (*B. subtilis* numbering: Asp241, Gln272, Leu308, Asn310, Asp339; [106]) are shown in bold. The two FTF catalytic residues shown in this alignment, the transition state stabilizer (SACB_BACSU Asp247) and the acid/base catalyst (SACB_BACSU Glu342) [106,123], are underlined.
SACB_STRMU – *Streptococcus mutans* SacB; SACB_STRSA – *Streptococcus salivarius* SacB; SACB_BACSU *Bacillus subtilis* SacB; INU_LACRE – *Lactobacillus reuteri* Inu; LEV_LACRE – *Lactobacillus reuteri* Lev; SACB_BACAM – *Bacillus amyloliquefaciens* SacB; LEV_CLOAC – *Clostridium acetobutylicum* levansucrase; LEV_GEOST - *Geobacillus stearothermophilus* levansucrase; LEV_LACJO - *Lactobacillus johnsonii* levansucrase precursor; LEV_LACSA - *Lactobacillus sanfranciscensis* levansucrase; LEV_PAEPO - *Paenibacillus polymyxa* levansucrase; UNK_BACLI *Bacillus licheniformis* unknown protein; FTF_ACTNA - *Actinomyces naeslundii* fructosyltransferase; BFF_ARTGL - *Arthrobacter globiformis* beta-fructofuranosidase; INU_LEUCI - *Leuconostoc citreum* inulosucrase. Asterisks (*) indicate conserved residues; (:) indicates that one of the following 'strong' group is conserved NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW; (.) indicates that one of the following 'weaker' groups is conserved: CSA, ATV, SAG, STNK, STPA, SGND, NDEQK, NDEQHK, NEQHRK, FVLIM, HFY.

3.4. Site-directed mutagenesis of the Lb. reuteri FTF putative Ca^{2+} binding site

The functional role of the conserved Asp339 residue in Ca^{2+} binding was studied by mutating the equivalent residues in the *Lb. reuteri* 121 Inu and Lev enzymes. Inu mutants

D520N, D520A, and the Lev mutants D500N, D500A were constructed, expressed in *E. coli* and purified. The Lev and Inu mutants displayed interesting differences. In each case a strong reduction in activity was observed over the whole range of temperatures tested (compare Figs. 1 and 4). Especially Inu activity had become severely reduced. In both Inu and Lev, the Asp to Ala mutations caused a stronger reduction in activity than the Asp to Asn changes. The stimulatory effects of Ca^{2+} ions had become much smaller in the mutant enzymes, especially in case of the Asp to Ala mutations (compare Figs. 1 and 4). Not surprisingly, compared to an Asn residue, the introduction of an Ala residue at this position thus had a much stronger effect. The activities of mutants Lev D500N and D500A, however, were still clearly stimulated by Ca^{2+} ions (Fig. 4B).

At 50°C, Inu mutant D520N was inactive in the absence of Ca^{2+}, with <1 % of wild-type activity remaining in the presence of 1 mM Ca^{2+} (specific activity reduced from 180 to 1 U/mg) (Fig. 4A). Mutant D520A displayed a low activity at 50°C in the absence of calcium (0.3 U/mg); its activity was clearly stimulated by the presence of calcium ions (3.6 U/mg). Mutant Inu D520N displayed a very severe reduction in affinity for Ca^{2+} binding, with a $K`_d$ value of 790 μM at 50°C (WT Inu at 50°C: $K`_d$ = 0.48 μM; approximately 1600-fold reduction in affinity). At lower temperatures, the Ca^{2+} dependency of Inu mutants D520N and D520A became reduced, also resulting in somewhat higher activities (11 and 6 U/mg, respectively). Inu WT incubated with 1 mM EDTA displayed the same relationship of activity with temperature as mutant D520N (without the presence of EDTA), albeit at a different level of activity (60 and 11 U/mg, respectively). Clearly, the mutations introduced in the Inu protein reduced its affinity for calcium binding, affecting both its stability and activity.

Characterization of Lev D500N revealed a clear but much smaller reduction in affinity for Ca^{2+} binding than observed for Inu D520N. Lev D500N displayed a $K`_d$ value of 260 μM at 45°C (Lev WT at 45°C: $K`_d$ = 6.9 μM; approximately 35-fold reduction in affinity). At 45°C, Lev mutants D500N and D500A were inactive in the absence of Ca^{2+} ions, with, respectively, 45 % and 15 % of wild-type activity remaining in the presence of 1 mM Ca^{2+} (Fig. 4B). In the presence of 1 mM Ca^{2+}, the Lev D500N and D500A mutants displayed highest activity at 40°C (53 and 19.5 U/mg, respectively). In the absence of Ca^{2+} ions, the optimal temperature for activity of the Lev D500N and D500A mutants was 30°C (22.5 and 11 U/mg, respectively) (Fig. 4B). Lev WT enzyme exhibited the same relationship for activity with temperature in the presence of 1 mM EDTA (12 U/mg, data not shown) as mutant D500N.

3.5. Conclusions

The Inu and Lev enzymes studied in this paper have the same bacterial origin (*Lb. reuteri* 121) and share high sequence similarity. Nevertheless, they display interesting differences in thermostability and calcium dependency. Lev activity becomes reduced at relatively low temperatures already (Fig. 1), but this protein is clearly more resilient,

largely recovering from activity loss during high temperature preincubations, even in the absence of calcium ions (Fig. 2). Mutations in the D520 (Inu) and D500 (Lev) residues in both cases resulted in strongly reduced activity levels (Fig. 4). Inu mutant D520N, however, was much more affected in its activity and affinity for calcium ion binding than Lev mutant D500N, with 1600 (at 50 °C) and 35-fold (at 45 °C) reduced $K`_d$ values, respectively. The structural basis for these differences remains to be elucidated.

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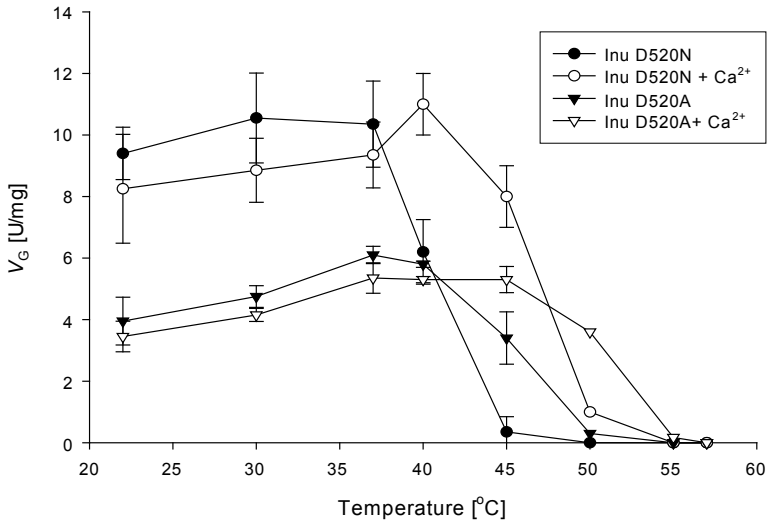

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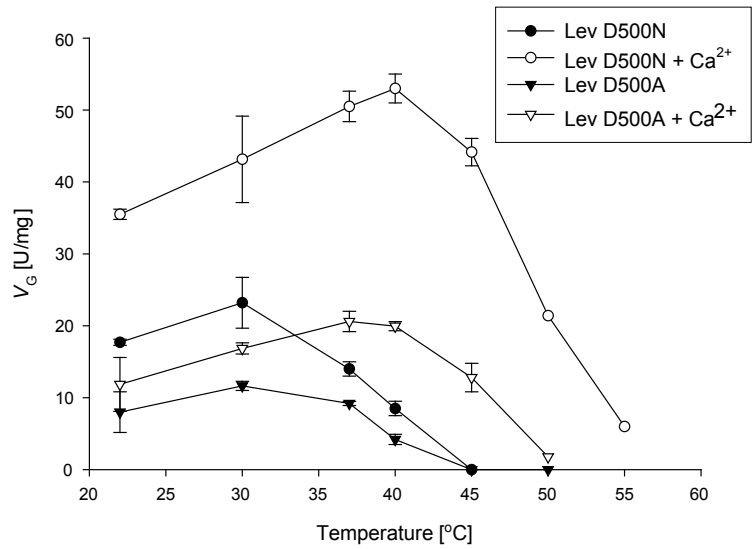

Figure 4. Relationship between specific activities (V_G) of the *Lb. reuteri* 121 Inu D520N, A (A) and Lev D500N, A (B) mutant enzymes and incubation temperature, measured in the absence or presence of 1 mM Ca^{2+} ions.

The data presented in this paper thus show that calcium ions have an important structural role in the *Lb. reuteri* 121 Lev and Inu proteins. Both proteins possess high-affinity Ca^{2+} binding sites. Residues D520 (Inu) and D500 (Lev), equivalent to D339 of *B. subtilis* levansucrase, are important for binding of the metal ion. The specific mutations D520N, D520A (Inu) and D500N, D500A (Lev) themselves, and/or the absence of Ca^{2+} caused by these mutations, may change the conformation of the strongly conserved DEIER motif, with the general acid catalyst [106,123], affecting catalysis and resulting in a strong reduction in FTF activities. Residues constituting this Ca^{2+} binding site [106] are completely conserved in most family GH68 enzymes from Gram-positive bacteria, suggesting that the stabilizing function of calcium ions is a general property of these proteins. This calcium-binding site appears to be absent in family GH68 enzymes of Gram-negative origin.

Chapter 4

The levansucrase and inulosucrase (fructosyltransferase) enzymes of *Lactobacillus reuteri* 121 catalyze (non-) processive transglycosylation reactions

L.K. Ozimek, S. Kralj, M.J.E.C. van der Maarel, and L. Dijkhuizen

Microbiology, in press

Abstract

Bacterial fructosyltransferase (FTF) enzymes belong to glycoside hydrolase family 68 (GH68) and catalyze synthesis of β-linked glycosidic bonds with net retention of the anomeric configuration and / or hydrolysis. These FTF activities result in: i) transglycosylation using sucrose, gluco- and fructooligosaccharides (oligosaccharide synthesis) or the growing fructan chain (polymerization) as acceptor substrates; ii) sucrose hydrolysis using water as acceptor.

The *Lactobacillus reuteri* 121 levansucrase (Lev) and inulosucrase (Inu) enzymes are closely related at the amino acid sequence level (86% similarity). Also the eight amino acid residues known to be involved in catalysis and / or sucrose binding are conserved. Nevertheless, these enzymes differ strongly in their reaction and product specificities, i.e. in β(2-6) vs. β(2-1) glycosidic bond specificity (resulting in levan and inulin synthesis, respectively), and in the ratio of hydrolysis vs. transglycosylation activities (resulting in glucose and fructooligosaccharides (FOS)/polymer synthesis, respectively).

Here we report a detailed characterization of the transglycosylation reaction products synthesized by the *Lb. reuteri* 121 Lev and Inu enzymes from sucrose and related oligosaccharide substrates. Lev converted sucrose mainly into a large levan polymer (processive reaction), whereas Inu mainly synthesized a broad range of FOS of the inulin-type (non-processive reaction).

Interestingly, both FTF enzymes also were able to utilize various inulin-type FOS (1-kestose, 1,1-nystose, 1,1,1-kestopentaose) as substrates, catalyzing a disproportionation reaction, not reported previously for bacterial FTF enzymes.

Based on this data we propose a model for the organization of the sugar binding subsites in these two *Lb. reuteri* 121 FTF enzymes that also explains their catalytic mechanism and differences in product specificities.

1. Introduction

Bacterial FTF enzymes catalyze transfer of the fructosyl residue from sucrose (and raffinose) to various acceptor substrates. FTF enzymes are known to catalyze two different reactions: i) transglycosylation using sucrose, gluco- and fructooligosaccharides (oligosaccharide synthesis) or the growing fructan chain (polymerization) as acceptor substrates ii) hydrolysis of sucrose when water is used as acceptor. These FTF enzymes belong to glycoside hydrolase family 68 (GH68) (http://afmb.cnrs-mrs.fr/CAZY/) [34]. They are β-retaining enzymes employing a double displacement mechanism that involves formation and subsequent hydrolysis of a covalent glycosyl-enzyme intermediate (a Ping Pong type of mechanism) [30,66,155].

Most bacterial FTFs known are levansucrases (Lev; EC 2.4.1.10), synthesizing fructan polymers composed of β(2-6) linked fructose units (levan) [58,161,187], and only limited information is available about bacterial inulosucrases (Inu; EC 2.4.1.9) producing β(2-1) linked fructan polymers (inulin) [9,121,139,189].

Only a few levansucrase enzymes have been characterized with respect to products synthesized from sucrose. The ratio between polymerization and oligosaccharide synthesis activities was found to differ significantly depending on the source of the enzyme. Hernandez *et al.* reported that levansucrase of *Bacillus subtilis* catalyzes the formation of a high molecular-mass levan without transient accumulation of oligofructan molecules [66]. This suggested that the growing polymer chain remained bound to the enzyme and that fructan chain elongation proceeded via a processive type of reaction. On the contrary, the levansucrases of *Gluconacetobacter diazotrophicus*, *Zymomonas mobilis,* and *Lactobacillus sanfranciscensis* mainly synthesized short fructooligosaccharides (FOS; kestose and nystose) from sucrose [43,66,84,87]. These enzymes thus may employ a non-processive type of reaction, involving release of the fructan chain after (virtually) each fructosyl transfer. The high resolution 3D structures of the *B. subtilis* levansucrase, mainly synthesizing large polymers, and the FOS synthesizing levansucrase from *G. diazotrophicus* recently have become available [104,106]. The active site architecture of both levansucrases carrying the same catalytic triad [123] is identical [104,106], and it appears very likely that both enzymes use a similar reaction mechanism. Nevertheless, it has remained unclear what structural features determine the polymerization vs. oligosaccharide synthesis ratio, and the use of a processive or non-processive mechanism for fructan chain growth in FTF enzymes.

Besides sucrose, also raffinose (GalGF), but neither kestose (GF2) nor nystose (kestotetraose, GF3), can be used as substrates by the FTF enzymes of family GH68 studied [66,179]. However, where studied, FTFs can use various mono- and oligosaccharides (e.g. maltose, maltotriose, raffinose, arabinose, xylose, and sucrose) as fructosyl acceptor substrates in their transglycosylation reactions with sucrose as donor

substrate (e.g. the levansucrases of *Aerobacter levanicum*, *B. subtilis* and *Lb. sanfranciscensis*) [170,176,194].

To date enzymes synthesizing inulin polymers have been identified in three Gram-positive bacterial species: *Streptococcus mutans* JC2 [9,139], *Leuconostoc citreum* CW28 [121] and *Lb. reuteri* 121 [189]. Thin layer chromatography (TLC) analysis of the reaction products of the inulosucrase of *S. mutans* JC2 revealed that, beside traces of sucrose, kestose, and nystose, a fructose-containing compound with a degree of polymerization (DP)>7 (FOS or/and inulin) was synthesized [69]. Further information about products synthesized from sucrose by inulosucrase enzymes is lacking at the moment.

Previously, we have isolated and characterized two FTF enzymes from *Lb. reuteri* 121. One of these enzymes is capable of synthesizing a levan (levansucrase) (Mr = 2×10^4 (97% of total) and Mr = $3\text{-}4\times10^6$ (3% of total)) [187] and the other one synthesizes an inulin (inulosucrase) (Mr = 10^7) [189]. These two FTF enzymes are very similar at the amino acid level (86 % similarity and 56 % identity, within 768 amino acids), both depend on Ca^{2+} ions for activity (to a different extent) and display similar, high temperature optima [122]. A characterization of the substrate and product specificities of the *Lb. reuteri* 121 Inu and Lev enzymes remained to be carried out.

Here we report a detailed characterization and comparison of the products formed by the *Lb. reuteri* 121 Inu and Lev enzymes from sucrose or inulin-type FOS as substrates. The data clearly show that Lev and Inu catalyze processive and non-processive reactions, respectively, and allow us to propose a model for the organization of the FTF active site and its acceptor substrate sugar binding subsites.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Escherichia coli strain Top10 (Invitrogen, Carlsbad, USA) was used for expression of the *Lb. reuteri* 121 inulosucrase (*inu*; GenBank accession number AF459437) and levansucrase (*lev*; GenBank accession number AF465251). Plasmid pBAD/myc/his/C (Invitrogen) was used for cloning and expression purposes. Plasmid carrying *E. coli* strains were grown at 37°C on Luria-Bertani medium [142] supplemented with 100 μg/ml ampicillin and 0.02 % (w/v) arabinose for *ftf* gene induction. The proteins were expressed in *E. coli* as constructs with a C-terminal truncation of 100 (Inu) or 32 (Lev) amino acid residues, and a C-terminal poly-histidine tag [187,189].

2.2. Purification of FTFs and enzyme activity assay

Inu and Lev of *Lb. reuteri* 121 were produced and purified by Ni-NTA affinity chromatography as described [189]. Purity was monitored by SDS-PAGE analysis. Enzyme concentrations were determined using the Bradford reagent (Bio-Rad, Munich, Germany) with bovine serum albumin as standard. Unless indicated otherwise, all enzymatic incubations were performed in the following reaction mixture: 50 mM sodium acetate buffer pH 5.4 supplemented with 840 mM sucrose and 1 mM $CaCl_2$. Activities of purified Inu (6.9 µg/ml) and Lev (9.6 µg/ml) enzymes were determined at 22°C, 37°C and 50°C. After preincubation of the assay mixture at the assay temperatures for 5 min, reactions were started by enzyme addition. Samples were taken every 3 min and used to determine the amount of glucose and fructose released from sucrose [186]. The amount of glucose formed reflects the total amount of sucrose utilized during the reaction (V_G). The amount of fructose (V_F) formed is a measure for the hydrolytic activity. The tranglycosylation activity was calculated by subtracting the amount of free fructose from glucose (V_G-V_F). One unit of enzyme activity is defined as release of 1 µmol of monosaccharide per min. Curve fitting of the data was performed as described previously using either the Michaelis-Menten formula, or the 3 parameter Hill formula [187,189]. Experiments were performed in duplicate (Fig. 1).

2.4. Analysis of reaction products

2.4.1. TLC analysis

In order to analyze the influence of temperature on the Inu and Lev oligomerization/polymerization reactions with sucrose, incubations were performed at 22 and 50°C using 12 and 120 U/ml of Inu, respectively, and 37 and 370 U/ml of Lev, respectively (final concentrations in the reaction mixtures) (Fig. 2). To investigate the product specificity of Inu and Lev with sucrose and various acceptor substrates, reactions were performed at 22 and 37 °C with 2 U/ml FTF (measured at 37 °C) (Figs. 3, 5 and 7). Incubations of Inu and Lev with kestopentaose only were performed at 22°C for 24 h (Fig. 5). Samples were taken at different time points (from 5 min to 24 h), diluted three fold in water and spotted (1 µl) on TLC plates (Silica gel 60 F_{254}; Merck Co., Ltd., Darmstadt, Germany). Solutions of 100 mM fructose (Merck), sucrose (Acros Organics, Geel, Belgium), 1-kestose (GF2) (Fluka Chemie AG, Buchs, Switzerland), 1,1-nystose (GF3) (Fluka Chemie AG), 1,1,1-kestopentaose (GF4) (Megazyme International Ireland Ltd., Wicklow, Ireland) were used as standards (0.5 µl spots). The plates were run once in a butanol:ethanol:water (5:5:3, v:v:v) mixture. Fructose-containing sugars were specifically stained using an urea spray [179]. Glucose-containing sugars were visualized by spraying with 50% sulfuric acid in methanol and heating at 110 °C [79].

Lev and Inu enzyme activities with 1-kestose, 1,1-nystose, 1,1,1-kestopentaose, and raffinose (α-D-galactopyranosyl (1-6) α-D-glucopyranosyl (1-2) β-D-fructofuranoside;

GalGF) (Sigma-Aldrich Co. Steinheim, Germany), and products synthesized, were analyzed by adding 840 mM of these sugars to the reaction mixture instead of sucrose.

2.4.2. High Performance anion-exchange chromatography analysis (HPAEC)

HP anion-exchange chromatography (Dionex, Sunnyvale, USA) was used to separate and / or determine the concentrations of fructose, glucose, sucrose, 1-kestose, 6-kestose, 1,1-nystose, and 1,1,1-kestopentaose after complete consumption of sucrose (end point conversion, incubation at 37°C for 24 h, with 840 mM sucrose, using 2 U/ml of FTFs) (Table 1). No standards were available for "unknown1" and bifructose (Fig. 4; Table 1). Therefore, the calibration curve for 1-kestose, representing the most closely related oligosaccharide available to us, was used to estimate the approximate concentrations of these two compounds. The Dionex analysis protocol used allowed us to calculate the concentration of most of the oligosaccharide products smaller than kestopentaose. The concentrations of all individual oligosaccharides were added up and, by subtracting this from the sucrose concentration used, the amount of products larger than kestopentaose was calculated (Table 1). Individual oligosaccharides and products larger than kestopentaose were expressed as a percentage of the total amount of sucrose initially present in the incubation. Unavoidably, there will be a discrepancy due to use of the 1-kestose calibration curve to determine concentrations of the two compounds (see above) and presence of other unknown oligosaccharides (especially for Lev) smaller than kestopentaose not taken into account in our calculations (Fig. 4; Table 1).

Separation of oligosaccharides was achieved by using a CarboPac PA1 anion-exchange column (250 mm x 4 mm; Dionex) coupled to a CarboPac1 Guard column (Dionex). The following gradient was used: eluent A (0 min, 100%); (10 min, 70%); (25 min, 60%); (80 min, 10%); (83 min, 0%); (91 min, 100%). Eluent A was 0.1 M sodium hydroxide and eluent B was 0.1 M sodium hydroxide in 0.6 M sodium acetate. Detection was performed with an ED40 electrochemical detector (Dionex) with an Au working electrode and an Ag/AgCl reference electrode. The amount of sucrose utilized during the reaction reflected total enzyme activity. The amount of fructose synthesized reflected hydrolytic enzyme activity. Total activity minus hydrolytic activity reflected transglycosylation enzyme activity (polymer and FOS formation). Based on these data the hydrolysis vs. transglycosylation ratio (end point conversion) was calculated.

The reaction products synthesized by Inu and Lev (2 U/ml at 37°C; activity measured with sucrose) with sucrose and kestopentaose (840 mM) as substrates were analyzed in the same way (after 24 h incubations), using Raftilose L85 as FOS standard and a 1:1 mixture of Raftiline ST-Gel and Raftiline HP (Orafti, Tienen, Belgium) representing chicory inulin standards. The Raftilose L85 standard is based on a partially hydrolyzed (fructanase-treated) chicory inulin, containing a range of GFn and Fn molecules (Figs. 4 and 6). Identification of the FOS in the standard was performed by preparative separation (HPAEC) followed by determination of monosugar composition of different fractions. Pure 6-kestose, 1-kestose and 1,1-nystose confirmed retention times of

short fructan components. Oligofructose molecules were identified as fractions with pure fructose. F2 was assigned as such, being the first pure fructose peak after fructose itself, F3 being the second pure fructose peak after fructose, and so on. Retention times and order were in agreement with [190].

3. Results

3.1. FTF enzyme activity

Kinetic studies at 22, 37 and 50°C revealed that the transglycosylation activity of *Lb. reuteri* 121 Inu increased gradually with increasing sucrose concentrations and that the enzyme could not be saturated by its substrate (Fig. 1, at 37°C) (see also [188]). At sucrose concentrations lower than 200 mM (at 37°C) hydrolysis was the main enzyme activity (not shown). At higher sucrose concentrations transglycosylation gradually increased, reaching 90% or more of total enzyme activity at 1.7 M sucrose.

Lb. reuteri 121 Lev mainly hydrolyzed sucrose at concentrations below 85 mM (at 37°C). Above 85 mM of sucrose, also the transglycosylation activity of Lev gradually became significant (not shown); both these Lev activities displayed Michaelis-Menten type of kinetics. Lev transglycosylation activity, however, never exceeded 50% of total enzyme activity (not shown). Compared to Inu, the transglycosylation activity of Lev

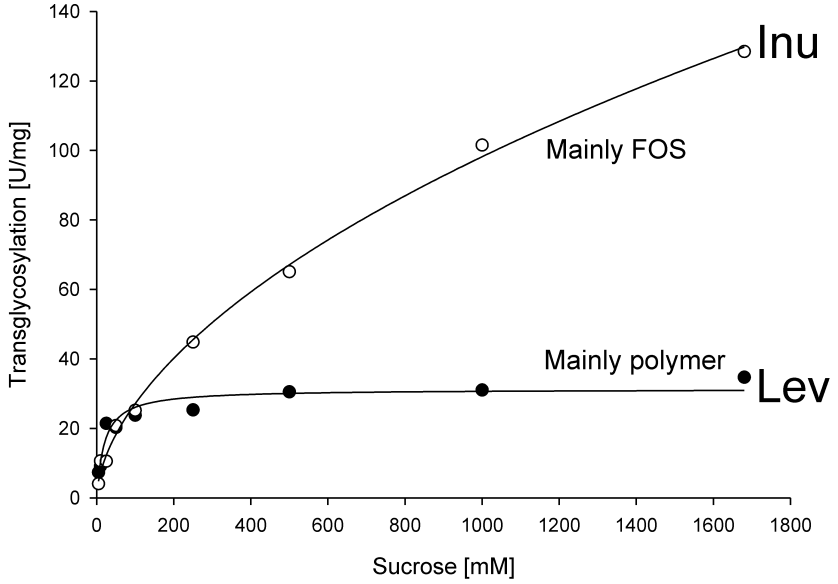

Figure 1. Comparison of the transglycosylation enzyme activities (initial rates at 37°C) at different sucrose concentrations using purified Inu (6.9 μg/ml) and Lev (9.6 μg/ml) proteins. Curve fitting of the data was performed as described previously using either the Michaelis-Menten formula, or the 3 parameter Hill formula [187,189]. Experiments were performed in duplicate (Fig. 1). Product analysis showed that Inu synthesized mostly FOS, whereas Lev synthesized mostly polymeric material.

varied with sucrose concentration in a relatively small range of concentrations only (Fig. 1). These amazing differences in enzyme kinetics prompted us to study the substrate and reaction product specificities of the *Lb. reuteri* 121 Lev and Inu enzymes in more detail.

3.2 TLC analysis of FTF products from sucrose

Both Inu and Lev of *Lb. reuteri* 121 have the highest specific activity towards sucrose utilization at 50℃ [122].

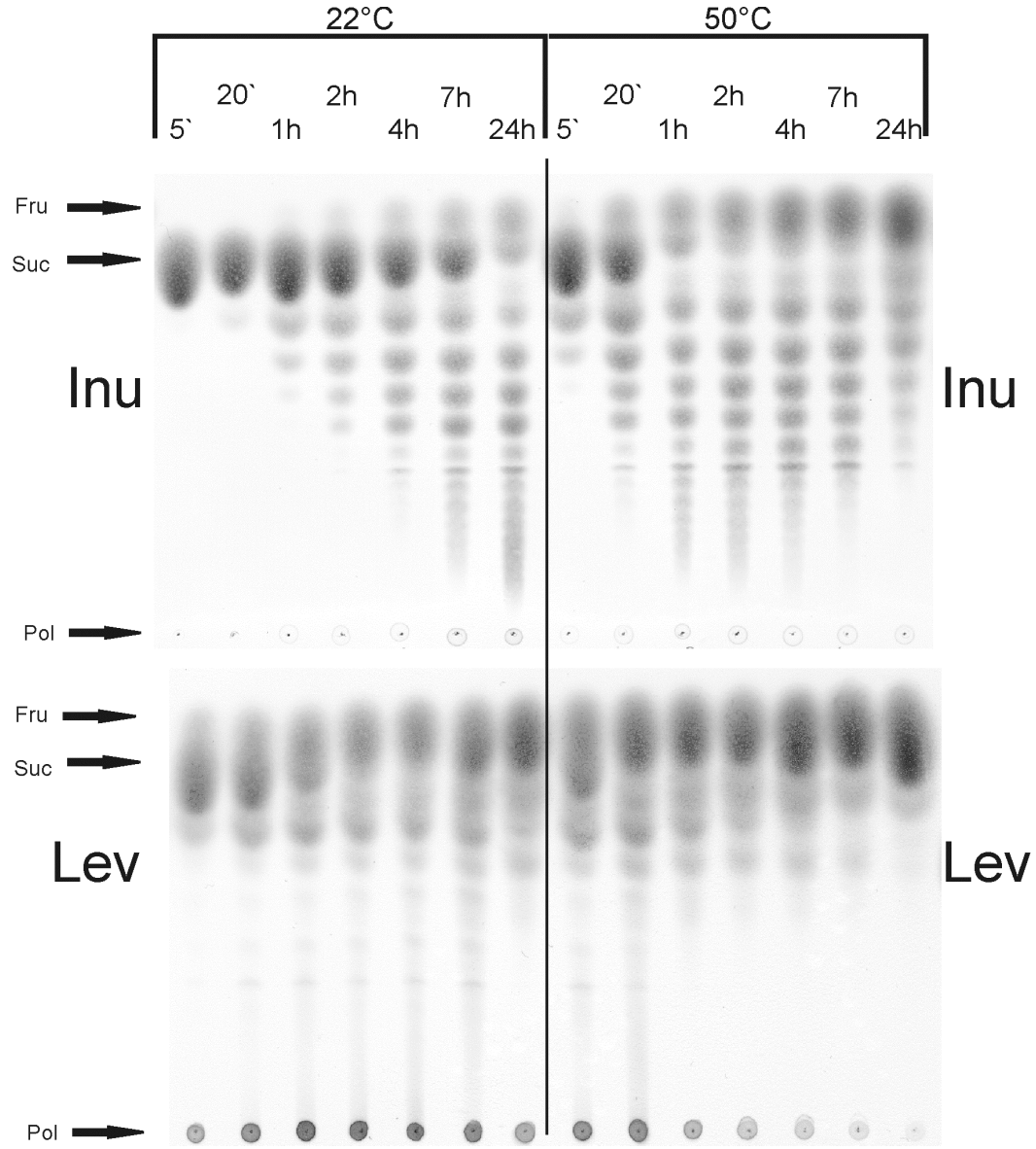

Figure 2. TLC analysis of the Inu and Lev products from sucrose (840 mM) at 22°C and 50°C at different time points (from 5 min to 24 h). The FTF enzymes were added as follows (activities measured with sucrose): Inu at 22°C – 12 U/ml, at 50°C – 120 U/ml; Lev at 22°C – 37 U/ml, at 50°C – 370 U/ml. Reactions were performed in the standard reaction buffer. The plates were run once in butanol:ethanol:water (5:5:3) mixture. Fructose-containing sugars were specifically stained with urea spray [179]. Fru - fructose; Suc - sucrose; Pol - polymer.

TLC analysis of the reaction products formed after incubation with sucrose showed that at 22°C only Inu initially synthesized a broad range of FOS (Fig. 2). During incubations at 50°C, the higher FTF enzyme activity resulted in much earlier depletion of sucrose. Inu subsequently started to degrade the larger size FOS (Fig. 2). Compared to Inu, Lev produced a relatively limited range of FOS at both 22 and 50°C, but synthesized much more levan polymer (see also [179]), detectable already at very early incubation times. At 50°C, after depletion of sucrose, also Lev started to degrade FOS and the levan polymer (Fig. 2).

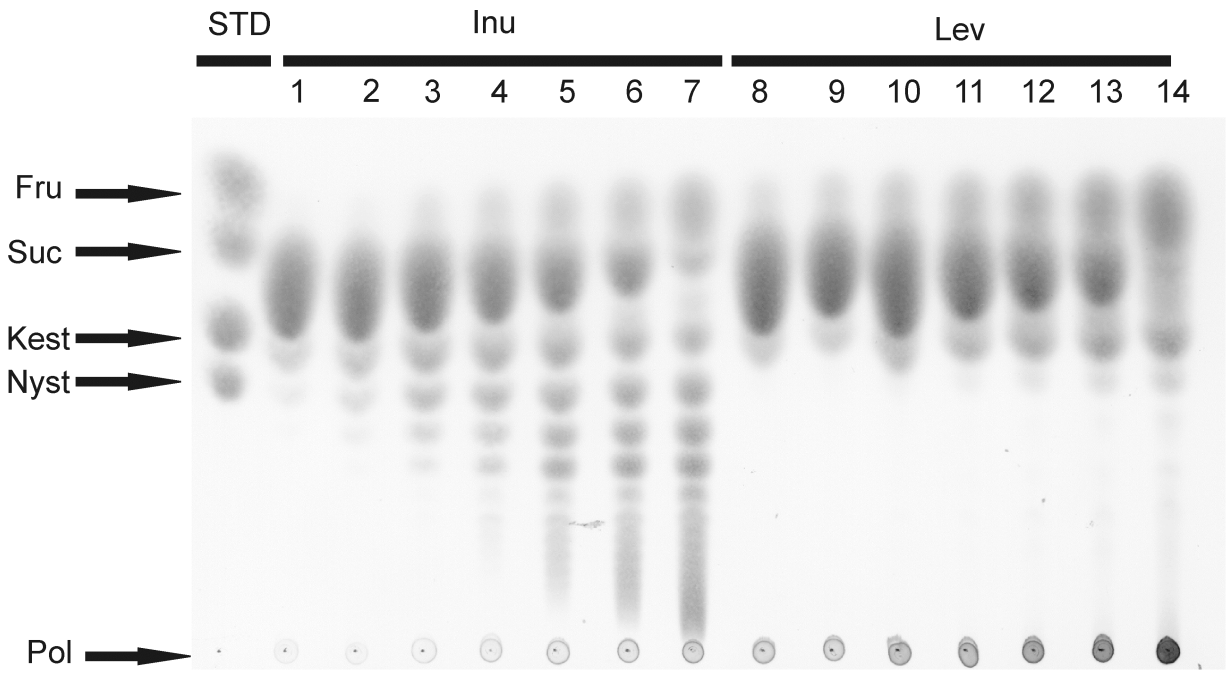

Figure 3. TLC analysis of the Inu and Lev products from sucrose (840 mM) at 22°C (using FTF enzymes at 2 U/ml, measured at 37°C). Samples were taken at different time points: Lanes 1 and 8: 5 min; 2 and 9: 20 min; 3 and 10: 1 h; 4 and 11: 2 h; 5 and 12: 4 h; 6 and 13: 7 h; 7 and 14: 24 h. The "STD" lane contains the following standards (from the top of the plate): fructose (Fru), sucrose (Suc), 1-kestose (GF2, Kest), 1,1-nystose (GF3, Nyst); Pol - polymer.

Incubations with Inu and Lev at similar activity levels allowed a more quantitative comparison (by TLC) of products formed by both enzymes from sucrose (Fig. 3). But again, Inu and Lev clearly differed synthesizing predominantly FOS and fructan polymer, respectively. Both enzymes synthesized similar amounts of kestose. Inu was able to synthesize nystose from the very early stage of the reaction; after 5 min of incubation traces of nystose were visible already. The nystose concentration remained constant once it reached a similar level as kestose (after 1 h), and synthesis of FOS of a larger size started. This scheme applied to all sizes of FOS synthesized during Inu incubations with sucrose; the presence of FOS of DP(n+1) could be detected only when DPn reached a certain threshold level. The TLC method allowed clear separation of oligosaccharides up to DP10. Inu also synthesized some inulin polymer (see also [189]), but only in relatively

minor amounts (Fig. 3). In contrast to Inu, Lev was able to synthesize FOS up to DP4 only. From the very early stage of the reaction, Lev synthesized much more fructan polymer than Inu (Fig. 3).

3.3 HP anion-exchange chromatography analysis of FTF products from sucrose

Anion-exchange chromatography analysis allowed identification and quantification of the oligosaccharide products formed by both FTF enzymes, and determination of the transglycosylation vs. hydrolysis product ratio. At the final stage of the reaction (end point conversion, after 24 h incubations), Inu and Lev had utilized (almost) all available sucrose (94 and 98%, respectively). Inu converted 82% of the sucrose into transglycosylation products and 18% of the sucrose was hydrolyzed. For Lev, this was 48 and 52%, respectively (Table 1). In case of Inu, 57% of the sucrose had been converted into FOS material larger than GF4 and 25% into FOS of GF4 or smaller (i.e. 1-kestose, 1,1-nystose, and into an unidentified product eluting after 9.5 min) (Table 1; Fig. 4).

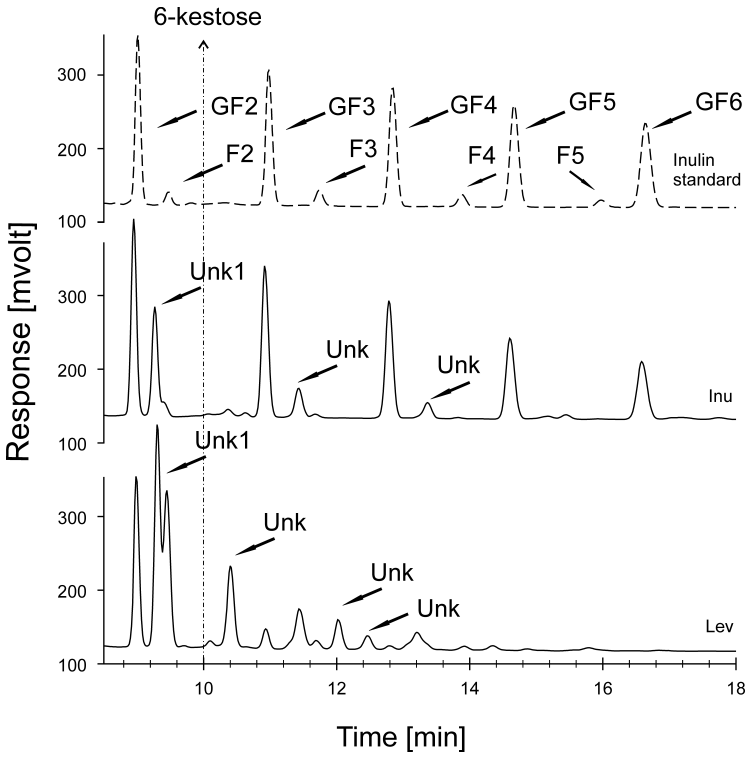

Figure 4. HP anion-exchange chromatography analysis of the Inu and Lev products from sucrose (840 mM) at 37°C during a 24 h incubation period.(using FTF enzymes at 2 U/ml). Raftilose L85 was used as FOS standard and a 1:1 mixture of Raftiline ST-Gel and Raftiline HP was used as inulin standard. Known oligosaccharides (GFn and Fn with β(2-1) linkages, and 6-kestose) are indicated. Unk = Unknown oligosaccharides.

The amount of FOS decreased gradually with increasing degree of polymerization, with DP15 representing the largest size FOS detected with HPAEC (not shown in Fig. 4). In case of Lev, only 28% of the transglycosylation products were larger than GF4

(virtually all levan polymeric material) and 20% was FOS smaller than GF5 (i.e. 1-kestose, 1,1-nystose, bifructose) (Table 1; Fig. 4). Lev synthesized several additional products that could not be identified with the inulin standards used (GFn and Fn molecules containing β(2-1) linkages).

The largest FOS product made by Lev was DP4-5 (Figs. 3 and 4). The main FOS synthesized from sucrose by Lev (7.4% of total; Table 1) eluted after 9.5 min, was not an inulin-type oligosaccharide, and remained unidentified. 6-Kestose is a very likely intermediate in levan (with β(2-6) glycosidic bonds) synthesis, but did not accumulate at detectable levels (Fig. 4). 6-Kestose thus may be used very efficiently for Lev polymer synthesis, reflecting the Lev processive reaction mechanism (see Discussion).

Table 1. Anion exchange chromatography analysis of the reaction products from sucrose (840 mM) (expressed as percentage of total amount of sucrose converted) of Inu and Lev (37°C, 24 h, 2 U/ml FTFs). [#] Lev synthesized additional unknown oligosaccharides (Fig. 4) not taken into account, therefore this value will be an overestimation.

	Inu	**Lev**
hydrolysis	18.3	51.6
transglycosylation	81.7	48.4
1-kestose	6.7	5.4
unknown-1	3.3	7.4
bifructose	0.0	6.0
1,1-nystose	9.2	1.7
1,1,1-kestopentaose	5.5	0.0
>kestopentaose	57	28[#]

3.4. Substrate and product specificity of Inu and Lev enzymes

Both Inu and Lev converted not only sucrose but also 1-kestose, 1,1-nystose, and 1,1,1-kestopentaose into various transglycosylation products. In case of Inu, TLC analysis of the reaction products revealed that, independently of the substrate used, a very similar range of oligosaccharides was synthesized (Fig. 5). All substrates were converted into small amounts of fructose and into considerable amounts of FOS (up to DP7 and larger). Small amounts of polymer were synthesized only from sucrose and 1-kestose (Fig. 5).

Lev converted all sucrose but some 1-kestose, 1,1-nystose, and 1,1,1-kestopentaose substrate remained after a 16 h incubation period (Fig. 5). The product specificity of Lev varied significantly depending on the substrate used. The amount of fructose released decreased with increasing size of the oligosaccharide substrates, suggesting that Lev has a lower hydrolytic activity with these larger inulin-type substrates. Lev converted these GFn substrates mainly into GF(n ± 1) and into small amounts of GF(n ± 2). The data thus show that both *Lb. reuteri* 121 FTF enzymes are able to catalyze a disproportionation type of reaction with 1-kestose, 1,1-nystose and 1,1,1-kestopentaose. Interestingly, Lev synthesized polymer from sucrose, and not from substrates larger than sucrose (Fig. 5).

The Inu and Lev products synthesized from kestopentaose (GF4) were also analyzed by anion-exchange chromatography (Fig. 6). Again, more kestopentaose remained in incubations with Lev than with Inu (after 24 h). Inu synthesized a broad range of FOS (DP3-7) from kestopentaose (Fig. 6). The main products synthesized were kestose, nystose, and GF5, apparently all of the inulin-type. Also Lev synthesized inulin type nystose in significant amounts, and some GF5 (Fig. 6). Both Inu and Lev also produced a range of products that could not be identified with the inulin-type FOS standards used, including the main product made by Lev (made in lower amounts by Inu as well, eluting after 13.5 min). A comparison with the standards shown in Fig. 4 shows that it is unlikely that these are unbranched Fn products of the inulin type. The exact identity of these oligosaccharides remains to be analyzed in future.

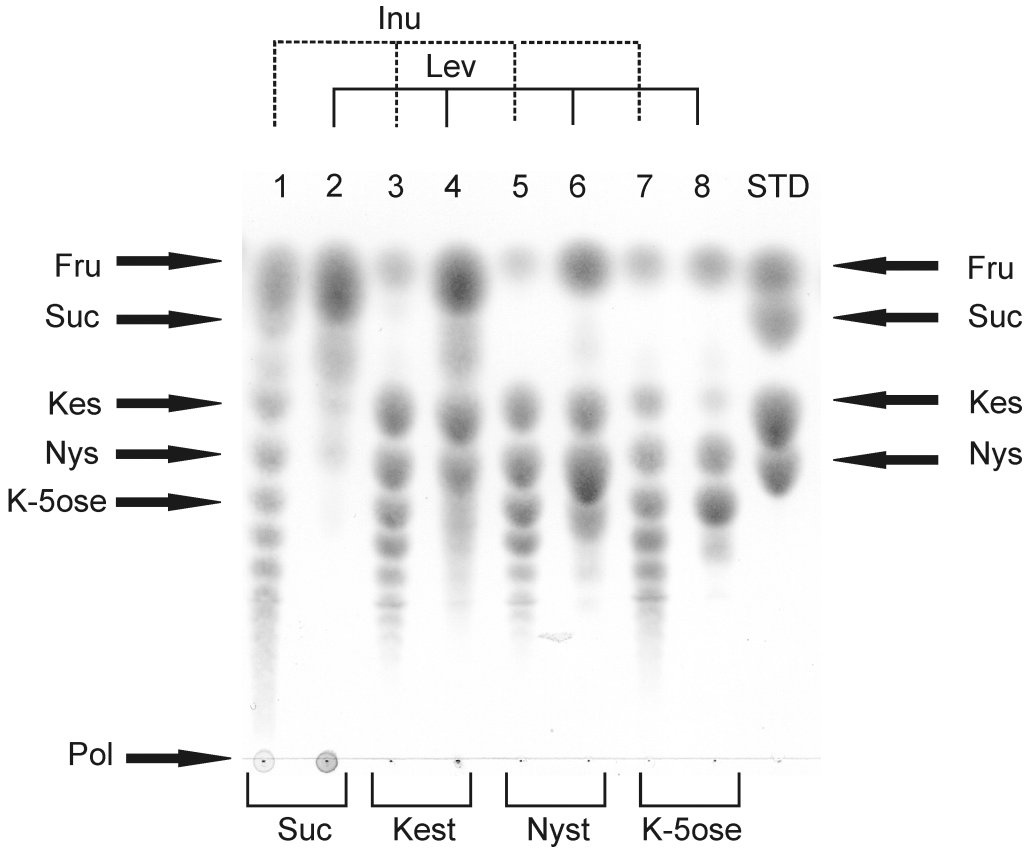

Figure 5. TLC analysis of the Inu and Lev products from different substrates (840 mM) at 22°C during a 16 h incubation period (using FTF enzymes at 2 U/ml, measured at 37°C). Odd and even numbers represent Inu and Lev samples, respectively. Enzymes were incubated with the following substrates: sucrose (lanes 1 and 2), 1-kestose (lanes 3 and 4), 1,1-nystose (lanes 5 and 6), and 1,1,1-kestopentaose (lanes 7 and 8). The "STD" lane contains the standards indicated.

Utilization of raffinose as fructosyl-donor and / or acceptor substrate for FTFs of *Lb. reuteri* 121 was subsequently tested. When incubated with raffinose alone (840 mM), both enzymes were able to synthesize fructosylraffinose (most likely GalGF2), but only Inu synthesized a whole range of larger oligomers (up to GalGF6) and some polymeric material (Fig. 7). The large amount of free fructose and the small quantities of oligosaccharides synthesized by Lev indicated that this enzyme has relatively high

hydrolysis and low transglycosylation activities with raffinose compared to sucrose. The additional presence of sucrose (200 mM) resulted in an even further extended range of GalGFn oligosaccharides synthesized by Inu, and in accumulation of more fructose by the action of Lev (Fig. 7).

4. Discussion

Although the *Lb. reuteri* 121 Inu and Lev enzymes are highly similar at the amino acid sequence level, strong differences were observed in their reaction kinetics with sucrose (Fig. 1). Aiming to understand the molecular and structural basis for these differences, we analyzed the transglycosylation products synthesized by these enzymes using sucrose and related short inulin-type FOS as substrates.

The Lb. reuteri *FTF enzymes catalyze processive and non-processive reactions*

Van Hijum *et al*. previously reported synthesis of inulin-type FOS from sucrose by Inu, but FOS larger than nystose were not observed when 0.1 U/ml of Inu enzyme was incubated with 250 mM sucrose at 37°C [189]. Our present studies revealed that Inu very efficiently synthesized inulin-type FOS of DP15 and larger when using relatively high sucrose and high Inu enzyme concentrations. In time, the FOS synthesized gradually increased in amount and size (Figs. 2-3). Inu apparently used the oligosaccharides synthesized as acceptor substrates in further transglycosylation reactions with sucrose as donor substrate. The characteristic TLC ladder-like pattern obtained for the Inu products synthesized in time (Figs. 2-3) showed that elongation of FOS is a non-processive reaction. For Inu, shorter FOS thus are better fructosyl acceptor substrates than longer ones. With increasing sucrose concentrations the range and concentration of synthesized FOS increased, resulting in accelerating total reaction velocity, also explaining the non-Michaelis-Menten reaction kinetics observed for Inu (Fig. 1).

Analysis of the reaction products synthesized by Lev from sucrose revealed a processive reaction, with synthesis of polymer evident very early on, and with a much smaller range of FOS synthesized. Lev can be saturated by its substrate sucrose, resulting in Michaelis-Menten type of kinetics (Fig. 1). The larger FOS molecules that are synthesized as intermediates in levan formation apparently remain bound to the Lev protein, and are directly used in further chain elongation steps, resulting in their rapid further processing.

FTF synthesis of inulin- and levan-like FOS

The data presented in this paper show that the FTF enzymes of *Lb. reuteri* 121 differ strongly in product spectrum from sucrose. Inu catalyzes synthesis of mostly β(2-1) linked FOS and a relatively low amount of inulin polymeric material (this study, see also

[189]). Lev synthesizes relatively more of a large polymer of the levan type (this study) containing almost exclusively (>95%) β(2-6) linkages (see also [186]), plus small amounts of β(2-1) FOS, and a range of unidentified molecules that may constitute β(2-6) FOS (Table 1, Figs. 2 and 3). The ability of levansucrase enzymes to synthesize β(2-1) and β(2-6) linked FOS has been reported before [48,84,166]. The *B. subtilis* C4 levansucrase synthesized a variety of FOS containing β(2-1) and β(2-6) linkages [48]. The main FOS synthesized was identified, as 1-kestose, while 6-kestose, natural primer for levan synthesis, was not detected. Euzenat *et al.* suggested that levansucrase produced 1-kestose that cannot be elongated further, resulting in its accumulation; 6-kestose may also be synthesized but does not accumulate and is rapidly used as acceptor substrate for levan synthesis [48]. Also the *Lb. reuteri* Lev did not accumulate 6-kestose, probably for the same reasons, and efficiently produced a levan polymer. The *Lb. reuteri* Lev enzyme also synthesized a range of FOS molecules from sucrose that remained unidentified (Table 1; Fig. 4). Also bifructose and 1-kestose were synthesized, however, suggesting that *Lb. reuteri* Lev, similar to *B. subtilis* levansucrase, exhibits a relatively low specificity in FOS linkage type formation. On the contrary, Inu of *Lb. reuteri* 121 displayed a higher specificity, synthesizing a range of inulin-type FOS (Fig. 4).

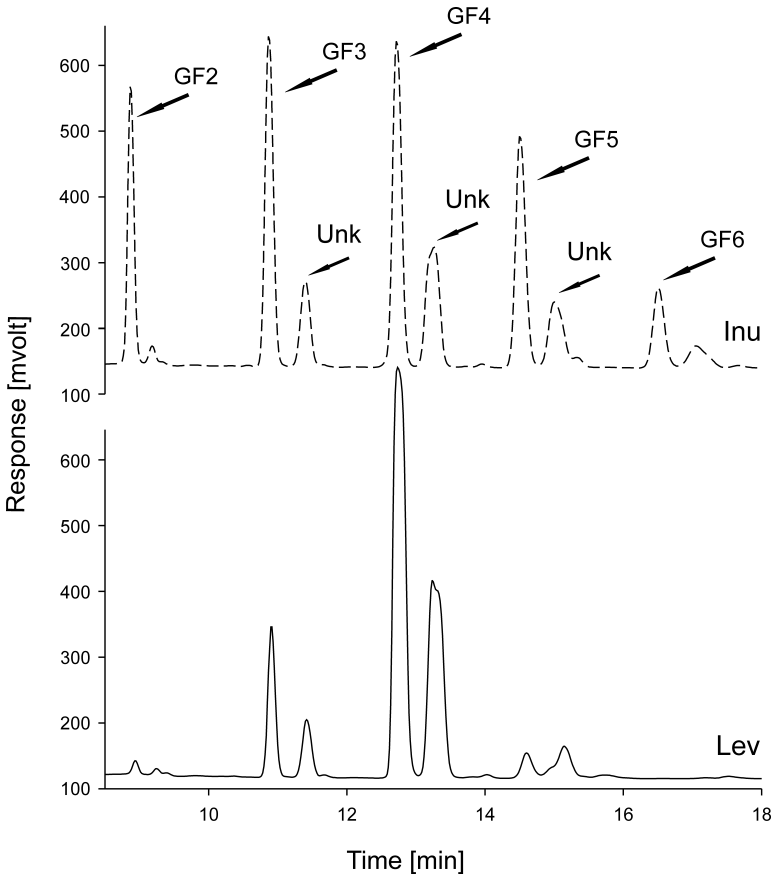

Figure 6. HP anion-exchange chromatography analysis of the Inu (dashed line) and Lev (solid line) products from 1,1,1-kestopentaose (840 mM) at 37°C during a 24 h incubation period (using FTF enzymes at 2 U/ml; activity measured with sucrose). Known oligosaccharides (GFn with β(2-1) linkages) are indicated. Unk = Unknown oligosaccharides.

Model for the organization of the FTF sugar binding subsites

The amino acid residues of subsites -1 and +1 in the active center of FTF enzymes (nomenclature according to Davies *et al.* [37]), directly interacting with sucrose, have been identified based on the 3D structures of *B. subtilis* levansucrase with bound sucrose [106] and several mutagenesis studies with FTF enzymes [25,200]. The catalytic triad in FTF enzymes of family GH68 previously have been identified as residues D86, E342, and D247 (numbering for the *B. subtilis* levansucrase), respectively acting as the catalytic nucleophile, the acid/base catalyst, and the transition state stabilizer [106,123]. Subsite -1 is formed by residues W85 and D86, and residues R246 and D247, part of the "RDP" motif that is highly conserved in all members of family GH68 [12]. Residues R360, E340, and R246 (part of the "RDP" motif) form subsite +1. These 7 residues, known to be involved in catalysis and / or interacting with the bound fructose and glucose at the -1 and +1 subsites, are completely conserved in the Lev and Inu enzymes of *Lb. reuteri* 121.

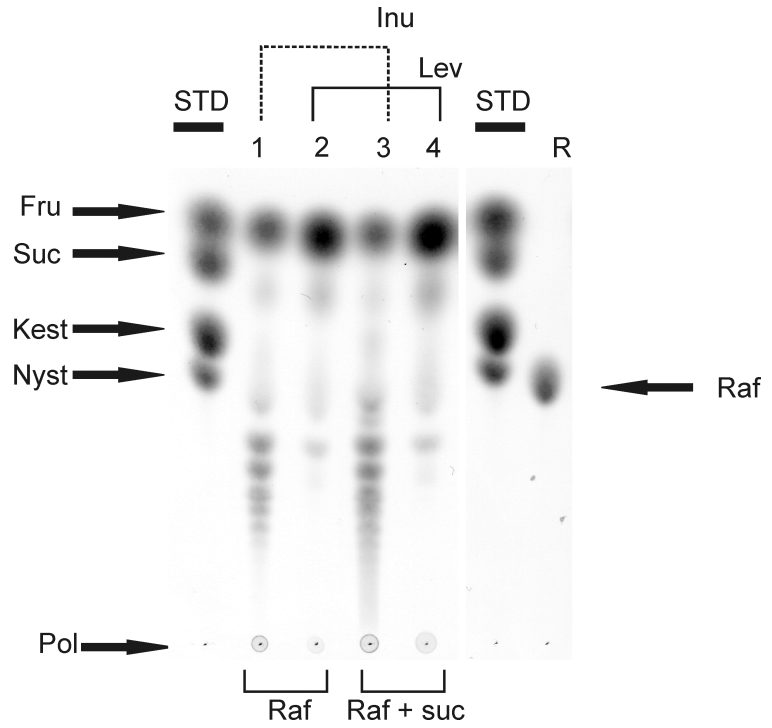

Figure 7. TLC analysis of the Inu and Lev products from raffinose (840 mM) (lanes 1 and 2) and raffinose (840 mM) plus sucrose (200 mM) (lanes 3 and 4) at 37°C during a 24 h incubation period (using FTF enzymes at 2 U/ml, activity measured with sucrose). Lanes labeled STD (containing standards), and 1 to 4 were stained with an urea spray (specific against fructose-containing sugars) [179] and the last two lanes (STD and R) were stained with a spray also visualizing glucose [79].

The FTF -1 subsite is highly specific for accommodating fructose units [30,66,155], whereas the +1 subsite is more flexible, exhibiting affinity for both glucose (binding of sucrose, raffinose) as well as for fructose (binding sucrose as an acceptor substrate during transglycosylation) (this study) (Fig. 8). Upon complex formation with sucrose the subsite +1 is occupied by a glucosyl moiety. As there is only one funnel-like channel leading

towards the active site [106], we propose the following reaction mechanism: sucrose enters the active site and occupies the -1 and +1 subsites. Its glycosidic bound is cleaved, a covalent fructosyl-enzyme intermediate is formed at -1 [24], and glucose is released through the channel. Subsequently, water may enter the active site, react with the fructosyl-enzyme intermediate, resulting in hydrolysis and release of fructose (Fig. 8B). Alternatively, a second sucrose acceptor substrate enters the active site, binds to the +1 and +2 subsites and reacts with the fructosyl-enzyme intermediate at -1, resulting in FOS formation (Fig. 8B). Further transglycosylation reactions may occur, resulting in chain elongation and polymer formation (Fig. 8C). A similar mechanism has been proposed previously for amylosucrase (family GH13) of *Neisseria polysaccharea* that catalyzes transfer of a D-glucopyranosyl moiety from sucrose onto an acceptor molecule [108].

The *Lb. reuteri* Inu and Lev enzymes both use sucrose, kestose, and raffinose as acceptor substrates (Figs. 5-7), pointing to further similarities in acceptor substrate binding sites. But Inu and Lev clearly differ in their ratio of hydrolysis versus transglycosylation activities, and in their ratio of synthesis of FOS versus fructan polymer, inulin and levan, respectively.

Since the residues constituting the –1 and +1 binding sites in Inu and Lev thus are virtually identical, unidentified structural differences in further acceptor substrate binding subsites must exist, responsible for these differences in Lev and Inu product specificity.

FTF enzyme catalyzed disproportionation reaction

The *Lb. reuteri* 121 Inu enzyme converted FOS (DP3-DP5 of the inulin type) into the same range of FOS as observed with sucrose (DP3 up to DP7 and larger) (Fig. 5). Two mechanisms may explain these observations: (i) oligosaccharides are degraded by hydrolysis of the terminal fructose units until sucrose is formed and sucrose is used as a substrate for transglycosylation; (ii) oligosaccharides are directly used as fructose donor and acceptor substrates, involving a disproportionation type of reaction, resulting in the first step in synthesis of GF(n±1) from a GFn substrate. In a second step, multiple reactions will occur. The relatively small amounts of free fructose released by Inu (Fig. 5) and the products range synthesized by Inu from kestopentaose (Figs. 5 and 6) indeed shows that Inu catalyzes a disproportionation reaction with these FOS (Fig. 8D and E). Our data indicate that FTF enzymes only have one donor subsite (-1). This correlates with the geometry of the active site of SacB from *B. subtilis*, showing a salt-bridge between E342 and R246 in the 3D structure, possibly disrupting further -2 and -3 subsites [106,123]. A similar situation has been observed in the amylosucrase of *N. polysaccharea* [2].

Lev less efficiently utilized FOS of the inulin-type, with more of the substrates remaining after 16 h of incubation (Figs. 5 and 6). The main products of the reaction of Lev with these inulin-type of FOS was FOS(n ± 1) plus fructose (Figs. 5 and 6). It thus appears that also Lev catalyzed a disproportionation reaction (Fig. 8D and E). This has not been reported previously for bacterial FTF enzymes. Both Inu and Lev thus cleave the

β(2-1) linkage at the non-reducing end of a fructosyl-oligosaccharide donor and transfer the fructosyl unit to the non-reducing end of another fructosyl-oligosaccharide acceptor. A similar scheme has been proposed for amylosucrase (family GH13) of *N. polysaccharea* [2]. The ability of FTF enzymes to catalyze disproportionation reactions undoubtedly will be of interest for testing inulin hydrolysates as fructosyl donors for oligosaccharide and fructo-conjugate synthesis.

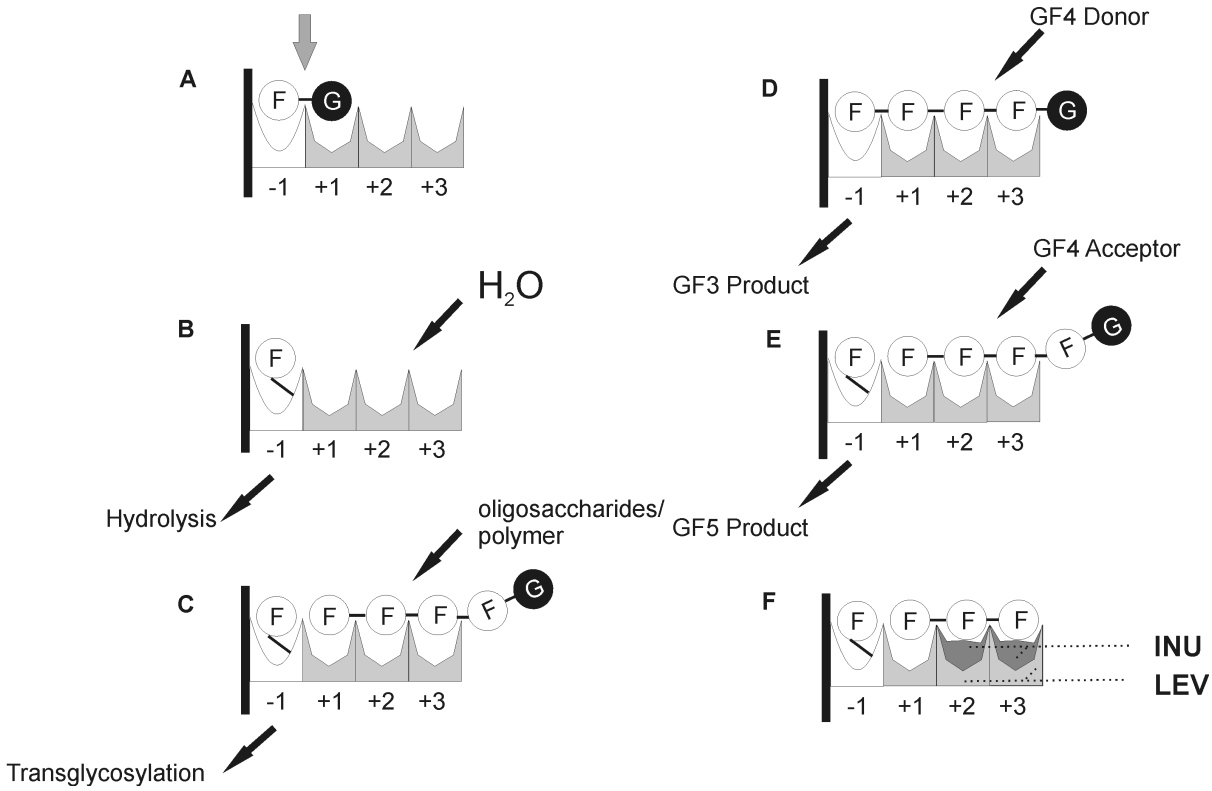

Figure 8. Schematic representation of the reaction sequences occurring in the active site of FTFenzymes. The donor and acceptor subsites of FTF enzymes are mapped out based on the available 3D structures [104,106] and data obtained in the present study. Binding of sucrose to subsites –1 and +1 (A) results in cleavage of the glycosidic bond, and formation of a (putative) covalent intermediate at subsite -1 (B). Depending on the acceptor substrate used, **hydrolysis** (with water) or **transglycosylation** reactions may occur (with oligosaccharides or the growing polymer chain, resulting in FOS synthesis or polymer synthesis, respectively) (B and C). The *Lb. reuteri* 121 FTF enzymes also catalyze a **disproportionation** reaction with inulin type of oligosaccharides. Kestopentaose (GF4) for instance is converted into GF3 and GF5 (D and E). The differences in affinity at the +2 and +3 subsites between Inu and Lev is reflected by a shallow (dark grey) (low affinity) and deep cleft (light grey) (high affinity), respectively (F). Sugar binding subsites are either shown in white (-1 subsite) reflecting specific and constant affinity for binding of fructosyl residues only, or in light/dark grey (+1, +2, +3 subsites) reflecting their ability to bind either fructosyl, glucosyl (with GFn substrate) or galactosyl (with raffinose) residues. The grey arrow indicates the position were cleavage/formation of glycosidic bond occurs. The black bar indicates the salt-bridge in FTF enzymes [E342 and R246 in SacB from *B. subtilis*] [104,106] possibly blocking further donor sugar binding subsites. F- fructose, G- glucose.

Fructan polymer synthesis requires the disaccharide sucrose as fructosyl donor substrate

Both Inu and Lev were unable to synthesize large polymers when 1,1-nystose or 1,1,1-kestopentaose were used as substrates. Only Inu synthesized a small amount of polymer from 1-kestose, and this actually may depend on sucrose availability, generated by kestose hydrolysis (Fig. 5). A likely explanation for the lack of fructan polymer synthesis from FOS is that polymerization, as a processive reaction, requires constant interaction of the enzyme with the growing polymer chain at subsites +2, +3 and further. Sucrose, due to its small size, apparently remains able to enter the active site and binds to -1 and +1 subsites of the enzyme, even when fructan polymer is bound at subsites +2, +3 and further. However, FOS larger than DP2 can serve as a fructosyl donor substrate only if the subsite +2 (in case of DP3), subsite +3 (in case of DP4) and further subsites (longer FOS) are accessible (Fig. 8). This competition between the growing polymer chain and fructosyl donors for the +2 and +3 subsites may result in a non-processive reaction type (disproportionation, oligomerization) and loss of ability to synthesize (larger) polymer. Whereas Lev was efficient in polymer synthesis (processive reaction), Inu displayed a clearly non-processive reaction with sucrose (Figs. 2 and 3). The Inu subsites +2, +3 and / or further, thus have a relatively low affinity for binding of the growing fructan polymer chain, resulting in its release after virtually every chain elongation with a fructosyl unit from sucrose (Fig. 8F). The overall effect of this is synthesis of FOS most of all, as observed with Inu (Figs. 2 and 3).

5. Conclusions

Characterization of two FTF enzymes of *Lb. reuteri* 121 (Inu and Lev) revealed that, although very similar in amino acid sequence, they differ significantly not only in the type of polymers synthesized (inulin and levan) but also in size distribution of FOS products synthesized from sucrose and related substrates. Inu synthesizes a whole range of well-defined inulin-type FOS of sizes reaching DP15 and larger, and a small amount of inulin polymer, whereas Lev produces mainly a levan polymer, and small quantities of short oligosaccharides probably containing (β(2-1) and / or (2-6) linkages. Synthesis of FOS is the main activity of Inu reflecting a much higher affinity of its acceptor binding subsites for shorter FOS than for longer ones. Lev mainly synthesizes a large polymer, most likely because its acceptor binding subsites have a much higher affinity for large polymers (DP5 and larger) than for short FOS. With Inu this results in a non-processive reaction with sucrose (after every fructosyl transfer the growing polymer chain is released from the enzyme). Lev catalyzes a processive type of reaction (the growing fructan chain remains bound to the additional acceptor binding subsites).

The availability of high-resolution 3D structures of FTF proteins with a bound long-chain acceptor substrates, and site-directed mutagenesis studies of the residues involved,

may serve to understand and predict the outcome of the transglycosylation reactions catalyzed by different FTF enzymes.

Acknowledgements

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Chapter 5

Engineering of the active site of the inulosucrase of *Lactobacillus reuteri*:

Polymer versus fructooligosaccharide synthesis

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Paper to be submitted for publication

Abstract

Bacterial fructosyltransferase (FTF) enzymes belong to glycoside hydrolase family 68 (GH68) and catalyze transglycosylation reactions with sucrose resulting in synthesis of fructooligosaccharides (FOS) and / or a fructan polymer. Significant differences in FTF enzyme product specificities can be observed, i.e. in the type of polymer (levan or inulin) synthesized, and in the ratio of polymer versus FOS synthesis.

The *Lactobacillus reuteri* 121 levansucrase (Lev) enzyme synthesizes mostly levan polymer (with β(2-6) linkages) and a little FOS [187]. The *Lb. reuteri* 121 inulosucrase (Inu) enzyme on the other hand produces a diverse range of FOS molecules and a minor amount of inulin polymer (with β(2-1) linkages) [188].

Analysis of the 3D structure of levansucrase (SacB) of *B. subtilis* revealed eight amino acid residues involved in sucrose binding. Sequence alignments showed that six out of these eight amino acid residues, including the catatlytic triad (D272, E523 and D424, Inu numbering), are completely conserved in GH68. The other three completely conserved residues are located at the -1 subsite (W271, W340 and R423). Our aim was to investigate the roles of these conserved amino acid residues in Inu (mutant proteins) with regard to activity and product profile.

Four Inu site-directed mutants at three different positions subsequently were constructed and characterized. Inu mutants W340N and R423H were virtually inactive, confirming the essential role of these residues in the Inu active site. Inu mutants W271N and R423K were less strongly affected in activity and displayed an altered FOS product pattern from sucrose, synthesizing a much lower amount of oligosaccharides and significantly more polymer. Interestingly, both these residues (R423 and W271) are located at the bottom of the FTF active site and constitute the -1 subsite. Our data show that the -1 subsite is not only important for substrate recognition and catalysis, but also plays an important role in determination of the size of products synthesized.

1. Introduction

Glycoside hydrolases (GH) are a large group of enzymes able to hydrolyze glycosidic bonds, displaying a great variety of protein folds and substrate specificities (http://afmb.cnrs-mrs.fr/CAZY/) [34]. They are divided into 100 different families based on their amino acid sequences. Since the amino acid sequence determines the structure of an enzyme, the catalytic mechanism appears to be conserved among the members of a given sequence-based family [36]. Evolutionary, structurally and mechanistically related families are grouped into "clans". Enzymes from families GH32 and 68 comprise the β-fructofuranosidase clan GH-J. Members of clan GH-J probably share three identical catalytic residues and use a similar reaction mechanism [193].

Family GH68 comprises bacterial fructosyltransferases (FTFs) that are retaining glycosidases. They catalyze transfructosylation reaction involving formation and subsequent cleavage of a covalent enzyme-substrate intermediate (Ping Pong type of mechanism) [30,66,155]. FTFs catalyze hydrolysis, when the acceptor that breaks the covalent fructosyl-enzyme bond is water, and / or transglycosylation reactions, when the bond is broken by either the growing fructan (polymerization) or oligosaccharide (oligomerization) chain. Depending on the type of linkages introduced in their products, FTFs are divided into inulosucrases (E.C. 2.4.1.9), synthesizing β(2-1) linked inulin, and levansucrases (E.C. 2.4.1.10), synthesizing β(2-6) linked levan. Family GH68 contains approximately 50 members (as of June 2005), which differ significantly, in the types of polymer (levan versus inulin) synthesized, and where studied, in the size of their transglycosylation products (FOS versus polymer), and / or their hydrolysis versus transglycosylation activity.

Family GH32 is much larger than GH68 and consists of enzymes with much broader substrate specificities and activities (invertases (EC 3.2.1.26); inulinases (EC 3.2.1.7); levanases (EC 3.2.1.65); exo-inulinases (EC 3.2.1.80); sucrose:sucrose 1-fructosyl transferases (EC 2.4.1.99); fructan:fructan 1-fructosyltransferases (EC 2.4.1.100)) (http://afmb.cnrs-mrs.fr/CAZY/).

To date the high-resolution 3D structures of the levansucrases from *Bacillus subtilis* (SacB) (with bound sucrose) and *Gluconacetobacter diazotrophicus* (LsdA), members of family GH68, have been solved [104,106]. Also three 3D structures of family GH32 representatives are available now: invertase from *Thermotoga maritima*, fructan 1-exohydrolase (1-FEH) from *Cichorum intybus* and exo-inulinase (EI) from *Aspergillus awamori*. [3, 193, 111]. All five enzymes possess a five-bladed β-propeller fold with a deep, negatively charged, central cavity. Their active sites are positioned at the end of this cavity with a funnel-like opening towards the molecular surface.

Inulosucrase enzymes have been characterized from three bacterial species: *Streptococcus mutans* JC2 [9,139], *Leuconostoc citreum* CW28 [121] and *Lactobacillus reuteri* 121 [189]. Where studied, all other members of family GH68 synthesize levan.

B. subtilis levansucrase catalyzes formation of high molecular mass levan without accumulation of FOS, whereas levansucrase of *G. diazotrophicus* synthesizes inulin-type FOS and low amounts of levan [66]. Comparison of the 3D structures of these two levansucrases showed that the structural determinants for synthesis of these different transglycosylation products cannot be identified at the moment [104]. Furthermore, the lack of 3D structures of inulosucrase proteins makes it impossible to identify structural features that determine the type of glycosidic bond, β(2-1) versus β(2-6), in the polymer (inulin versus levan) synthesized.

Previously, we have isolated and characterized two FTFs from *Lb. reuteri* 121. Lev synthesizes a high molecular mass levan [187], and Inu synthesizes inulin-type FOS and a small quantity of high molecular mass inulin [188] (see also Chapter 4). These two FTF enzymes are very similar at the amino acid level (86 % similarity and 56 % identity, within 768 amino acids), both depend on Ca^{2+} ions for activity (but to a different extent) and display high temperature optima [122] (See also Chapter 3). Using site-directed mutagenesis, we have also identified the catalytic triads in both enzymes [123] (Chapter 2).

Based on the available 3D structures and on alignment of amino acid sequences of family GH68 members (Figs. 1 and 2), we have identified 3 amino acid residues, conserved in all members of family GH68, as further targets for mutagenesis in the *Lb. reuteri* 121 Inu enzyme. None of these residues have been mutated before in members of GH68. Provided there is a high structural similarity between levansucrase and inulosucrase proteins, all three these residues (W271, R423 and W340; Inu numbering) should be located at the -1 subsite, interacting with the fructosyl unit of a bound sucrose (Fig. 1). Information about the functional roles of these conserved FTF residues is considered important as a first step to identify structural features responsible for product formation in FTF enzymes.

Here we report the characteristic properties of four mutant *Lb. reuteri* Inu proteins (W271N, W340N, R423H, and R423K). Based on analysis of their total enzyme activities, their transglycosylation activities, and the range of (FOS and polymer) products synthesized, mutations in all three Inu active site residues strongly affected total enzyme activity whereas mutations in two of these residues changed the amount of polymer and the size distribution of FOS synthesized.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Escherichia coli strain Top10 (Invitrogen) was used for expression of wild-type (WT) and mutant *Lb. reuteri* 121 inulosucrase (*inu*; GenBank accession number

AF459437) and WT levansucrase (*lev*; GenBank accession number AF465251). Plasmid pBAD/myc/his/C (Invitrogen) was used for cloning purposes. Plasmid carrying *E. coli* cells were grown at 37℃ on Luria-Bertani medium [142] supplemented with 100 μg/ml ampicillin and 0.02 % (w/v) arabinose for *ftf* gene induction. WT and mutant of Inu were expressed in *E. coli* as constructs with a C-terminal truncation of 100 amino acid residues, and a C-terminal poly-histidine tag as described [189]. The *bdri* construct was used for optimal Lev expression (see Chapter 3).

2.2. Molecular techniques

An alignment (SequenceLogo, http://www.bio.cam.ac.uk/seqlogo/) of 20 members of family GH68 was constructed, showing short regions in FTF enzymes with conserved amino acid residues (Fig. 2). Using site-directed mutagenesis, the following *Lb. reuteri* 121 Inu mutants were constructed: W271N, W340N, R423K, and R423H. Single mutations were introduced using the "megaprimer" method [143] and confirmed as described before (see Chapter 4). For site-directed mutagenesis (synthesis of "megaprimers") the following oligonucleotides were used in PCR reactions:
i-W271N: 5'- CTGGCCATGAATC**ATT**TACATCTAAAGG-3'; i-W340N: 5'-
CAGCTGATCCTGA**ATT**TTCTTGTGAAACC-3'; R423H: 5'-
CGATAATATTGCAATGC**A**TGATGCTCATG-3'; R423K: 5'-
CGATAATATTGCAATG**AAG**GATGCTCATG-3'.
Additionally, depending on the position of the mutated residue, one of the two flanking primers were used in combination with "megaprimers": i-pBADRV, 5`-
TCTGAGATGAGTTTTTGTTCGG-3`; pBADFD, 5`-
TCCTACCTGACGCTTTTTATCG-3`. The bases in bold indicate mutations introduced; i-: antisense primer.

2.3. Purification of FTF proteins

All proteins produced were expressed with a C-terminal histidine tag and purified by Ni-NTA affinity chromatography as described [189]. Purity was checked by SDS-PAGE. Enzyme concentrations were determined using Bradford reagent (Bio-Rad) with bovine serum albumin as standard.

2.4. FTF enzyme activity assay

Activities of the WT and mutant FTF enzymes were determined at 37°C in 50 mM sodium acetate reaction buffer pH 5.4, supplemented with 840 mM sucrose and 1 mM Ca^{2+}, using purified *Lb. reuteri* 121 enzymes, Lev WT (7.6 μg/ml protein), Inu WT (1.28 μg/ml) and Inu mutants: W271N (32 μg/ml), R423H (25.6 μg/ml), R423K (128 μg/ml), and W340N (32 μg/ml). After preincubation of the reaction mixture at the assay temperature for 5 min, reactions were started by enzyme addition. Samples were taken

every 3 min and used to determine the amount of glucose released from sucrose [186]. The amount of glucose formed reflects the total amount of sucrose utilized during the reaction (total activity, V_G). The amount of fructose formed is a measure of the hydrolytic activity (V_F). The amount of glucose minus the amount of fructose reflects the transferase activity (V_{G-F}). One unit of enzyme activity (U) is defined as the release of 1 μmol of monosaccharide per min.

2.5. Detection and quantification of FOS

Products synthesized were analyzed by thin layer chromatography (TLC, see Chapter 4). The following enzyme concentrations were used: W271N, Inu WT and Lev WT – 2 U/ml; R423H – 0.016 U/ml; R423K- 0.31 U/ml; W340N – 0.25 U/ml.

FOS synthesized by Inu WT and mutants W271N and R423K were also analyzed using HP anion-exchange chromatography as described (Chapter 4), following incubations of purified FTF enzymes in the reaction buffer containing 840 mM sucrose, at 37°C for 24 h. The following amounts of enzymes were used: 2.2 U/ml of Inu WT, 2.6 U/ml of mutant W271N and 0.2 U/ml of mutant R423K.

3. Results

3.1. Close-up view of the active site of FTF enzymes of family GH68

Elucidation of the high-resolution 3D structures of *B. subtilis* levansucrase and a sucrose-bound inactive mutant protein, and the G. *diazotrophicus* LsdA levansucrase protein, provided clear insights in the reaction mechanism of FTF enzymes [106]. Superposition of the available 3D structures revealed that the active sites of these enzymes show extensive overlap with each other's [193]. Based on these 3D structures, eight amino acid residues directly involved in binding of sucrose in the active site, and constituting the -1 and +1 sugar binding subsites (nomenclature according to Davies *et al.* [37]), were identified [106] (Fig. 1). Identical residues were found in the *Lb. reuteri* 121 Inu (Figs. 1 and 2) and Lev proteins (Fig. 2).

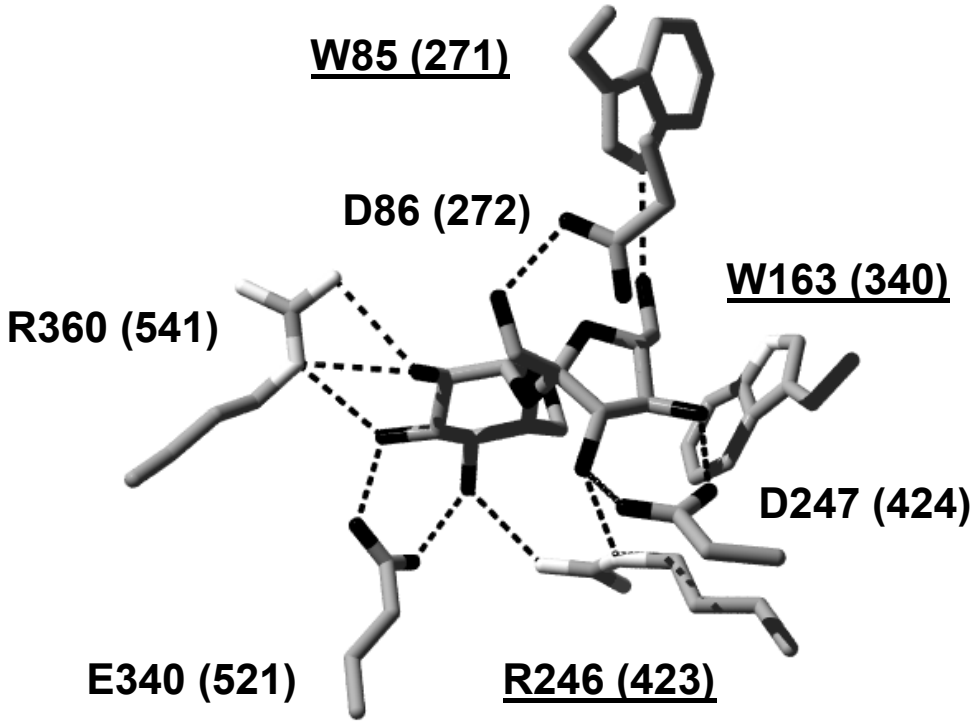

Figure 1. Close up view of the active site of mutant E342A of *B. subtilis* SacB levansucrase with a bound sucrose molecule (accession code: 1PT2). Figure was created using the SwissPdb Viewer (http://www.expasy.org/spdbv/) [59]. Hydrogen bonds are shown by dashed lines, based on [106]. Numbering of amino acid residues is based on *B. subtilis* SacB, with the numbering of *Lb. reuteri* Inu between parentheses. Residues that have been substituted in this study have been underlined

3.2. Sequence alignment of members of family GH68

Based on the available 3D structures and sequence alignment of family GH68 proteins, residues creating subsite -1 were identified in *B. subtilis* SacB, *G. diazotrophicus* LsdA and *Lb. reuteri* 121 Inu (Figs. 1 and 2). This subsite is constituted by the catalytic nucleophile (D86 in SacB, D135 in LsdA, and **D272** in Inu) [123], the neighboring tryptophane residue (W85 in SacB, W134 in LsdA, and **W271** in Inu), two residues located in the "RDP" motif that is conserved in most members of family GH68 (R246, D247 in SacB, R308, D309 in LsdA, and **R423**, **D424** in Inu), and a tryptophane residue bordering the sucrose binding pocket and located very close to the fructose moiety at -1 (W163 in SacB, W224 in LsdA, and **W340** in Inu). Inu residue Asp424 acts as transition state stabilizer [123]. Subsite +1 differs amongst different enzymes from family GH68 and is constituted by either an arginine or histidine residue (R360 in SacB, H419 in LsdA, and **R541** in Inu) (see below), either an glutamic acid or glutamine residue (E340 in SacB, Q399 in LsdA, and **E521** in Inu) located 2 residues upstream of the acid/base catalyst

(E342 in SacB, E401 in LsdA, and **E523** in Inu), and the arginine residue present in the "RDP" motif (also involved in formation of -1 subsite) (R246 in SacB, R308 in LsdA and **R423** in Inu). These differences at the +1 subsite distinguish between enzymes from Gram-positive (SacB and Inu) and Gram-negative (LsdA) bacteria but not between polymerizing (SacB) and oligomerizing (LsdA and Inu) nor inulin (Inu) / levan (SacB, LsdA) synthesizing enzymes.

Inu residues W271, W340, R423, conserved in all members of family GH68, were selected as targets for site-directed mutagenesis. To our knowledge none of these residues have been mutated and analyzed before in members of family GH68.

3.2. Activity of Lb. reuteri *121 Inu WT and active site mutants*

Previously, we have constructed and characterized Inu (and Lev) mutants modified in the three catalytic residues, E523Q (E503Q) (general acid/base catalyst), D272N (D249N) (catalytic nucleophile), D424N (D404N) (transition state stabilizer), resulting in at least 10,000-fold reduction in total activity [123].

The *Lb. reuteri* 121 Inu and Lev WT enzymes possess unusual high temperature optima for activity (V_G). Highest total activities were observed at 50°C [122], while the range of synthesized products increased at lower temperatures, and with relatively low activity units (Chapter 4). Independent of temperature, transglycosylation activity of Inu increased with sucrose concentrations, reaching 90% of total activity at 0.8 to 1.7 M sucrose. In order to optimize transglycosylation, reactions were performed at 37°C in the reaction buffer with 840 mM sucrose.

All Inu mutants showed strongly reduced V_G compared to Inu WT (20-370 fold), while the ratio between transglycosylation and hydrolysis remained virtually unchanged (Table 1) under the conditions tested. Mutants W271N, R423K, W340N, and R423H, displayed only 5.3, 1.4, 0.5, and 0.3% of total Inu WT activity, respectively. This reflects that also in Inu these residues make very important contributions, and are most likely involved in binding of the fructosyl unit of sucrose at the –1 subsite (W271, W340, R423) and the glucosyl unit of sucrose at the +1 subsite (R423) in the Inu active site (Fig. 1).

Table 1. Total, transglycosylation and hydrolytic activities (in U/mg of protein) of *Lb. reuteri* 121 Inu WT, Lev WT, and derived Inu mutants. Assays were performed at 37°C in a buffer supplemented with 840 mM sucrose. Samples were taken every 3 min; n.d – not determined.

enzyme	total	transglycosylation	hydrolysis	transglycosylation/hydrolysis
Inu	147	119	28	4.25
Lev	101	73	28	2.6
W271N	7.8	6.2	1.6	3.9
W340N	0.74	0.5	0.24	2
R423H	0.4	n.d.	n.d.	-
R423K	2	1.4	0.6	2.3

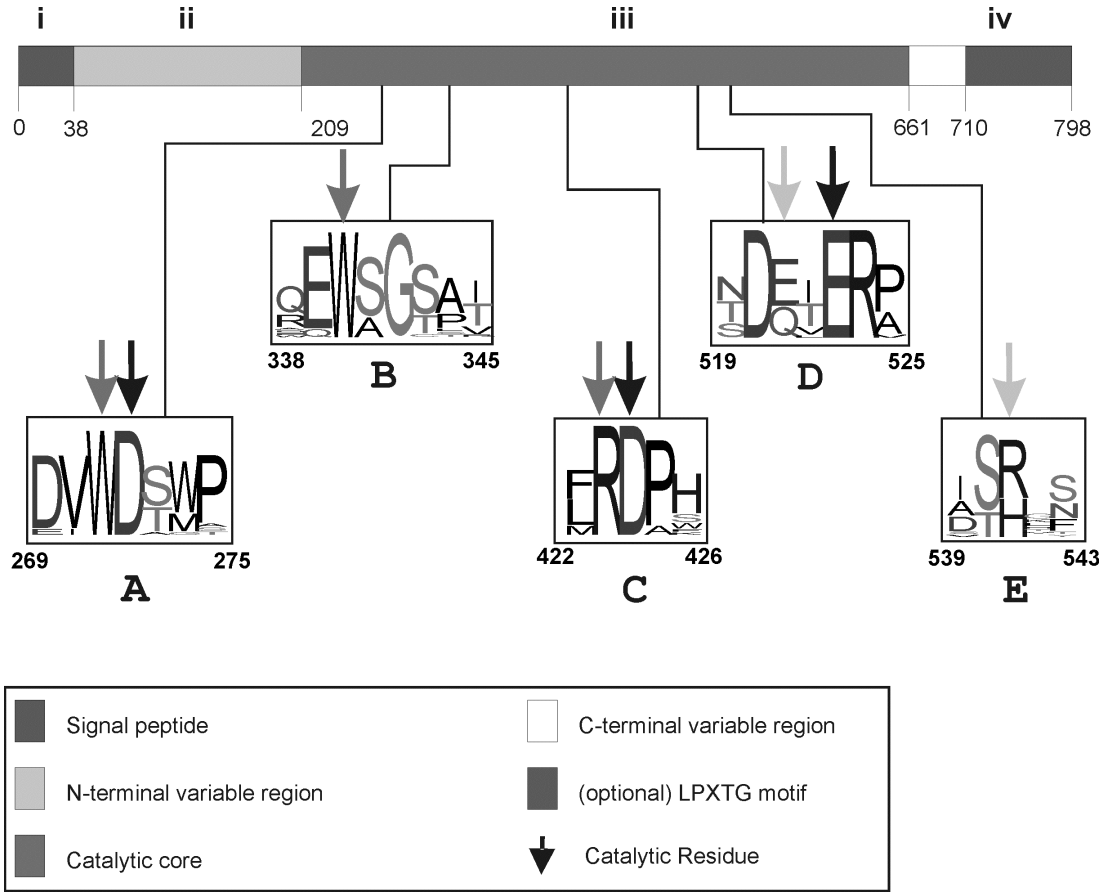

Figure 2. Schematic representation of family GH68 proteins. The *Lb. reuteri* inulosucrase (Inu) deduced amino acid sequence was used as template (AF459437). The four different regions shown are (i) N-terminal signal sequence; (ii) N-terminal variable region; (iii) catalytic core; and (iv) C-terminal variable region (which in some cases contains an LPXTG cell-wall anchor). Alignments (SequenceLogo, http://weblogo.berkeley.edu/) are shown of short regions in family GH68. Analysis of the 3D structure of levansucrase (SacB) of *B. subtilis* revealed eight amino acid residues involved in sucrose binding (see also Fig. 1). Sequence alignments showed that six out of these eight amino acid residues, including the catatlytic triad (D272, E523 and D424, Inu numbering), marked with black arrows [123] are completely conserved in GH68. The other three completely conserved residues are located at the -1 subsite (W271, W340 and R423), indicated with dark grey arrows. The other two residues involved in sucrose binding that are not completely conserved are indicated with light grey arrows. The following twenty amino acid sequences were used for the alignment: *Lactobacillus reuteri* Inu; *Bacillus subtilis* SacB; *Gluconacetobacter diazotrophicus* levansucrase; *Lactobacillus reuteri* Lev; *Lactobacillus sanfranciscensis* levansucrase; *Streptococcus mutans* SacB; *Streptococcus salivarius* SacB; *Geobacillus stearothermophilus* levansucrase; *Bacillus amyloliquefaciens* SacB; *Clostridium acetobutylicum* levansucrase; *Paenibacillus polymyxa* levansucrase; *Gluconacetobacter xylinus* levansucrase; *Zymomonas mobilis* levansucrase; *Pseudomonas syringae* levansucrase; *Rachnella aquatilis* levansucrase; *Erwinia amylovora* levansucrase; *Pseudomonas aurantiaca* levansucrase; *Leuconostoc citreum* inulosucrases; *Lactobacillus johnsonii* levansucrase precursor; *Leuconostoc mesenteroides* levansucrase.

3.3. Polymerization versus oligomerization

Lev WT, Inu WT and Inu mutant W271N were added at equal activity levels to the reaction mixture, allowing proper comparison of the reaction products by TLC (Fig. 3). TLC analysis showed that Inu WT synthesized a range of FOS up to at least DP9-10 and a small amount of inulin polymer, whereas Lev produced mainly (levan) polymer and smaller size FOS (mainly kestose and nystose) (see also Chapter 4). Compared to Inu WT, mutant W271N synthesized very similar amounts of FOS up to DP6, but clearly less of DP7-9. Interestingly, W271N synthesized more of the larger FOS (>DP10) molecules and polymer.

With the relatively low remaining activities of mutants W340N, R423K, and R423H, only substantially lower activity levels were available of these enzymes for incubation with sucrose in reaction mixtures. No sucrose conversion was detected with mutant R423H when using TLC analysis (24 h incubation; Fig. 3), although a low activity clearly was detected when using a more sensitive assay for glucose release from sucrose (initial rates; Table 1). Mutant W340N had lost ability to synthesize a range of FOS molecules and only synthesized small amounts of kestose and nystose; polymer formation was not detectable (Fig. 3). Mutant R423K still produced a relatively broad range of FOS molecules (in 24 h incubations), despite the lower activity levels of this enzyme used in the reaction mixture (only 0.3 U/ml compared to 2 U/ml for Inu WT) (Fig. 3). Also the amounts of FOS synthesized by mutant R423K were comparable (FOS up to DP6) between mutant and WT enzymes. Mutant R423K failed to produce FOS of DP6-10, but synthesized clearly more polymer and high DP FOS (>DP10) compared to WT Inu. With respect to the range of FOS and the amount of polymer synthesized, mutants W271N and R423K thus resembled each other clearly, strongly differing from Inu WT (Fig. 3).

3.4. HP anion-exchange chromatography analysis of FTF products from sucrose

HP anion-exchange chromatography analysis of the reaction products from sucrose allowed a detailed comparison of the product specificities of Inu WT and mutants W271N and R423K. With Inu WT, the amount of FOS synthesized was inversely proportional to the FOS size (Fig. 4A). The largest Inu WT FOS product detectable, with the method used, was DP18. In case of the mutant Inu enzymes this inverted relationship was only seen for oligosaccharides smaller than DP6. Mutant W271N synthesized FOS of at least DP20. The larger oligosaccharides were present in small amounts, visible through the whole elution profile (Fig. 4B). Mutant R423K synthesized FOS up to DP12 (Fig 4C), but in significantly lower amounts than in WT and mutant W271N samples.

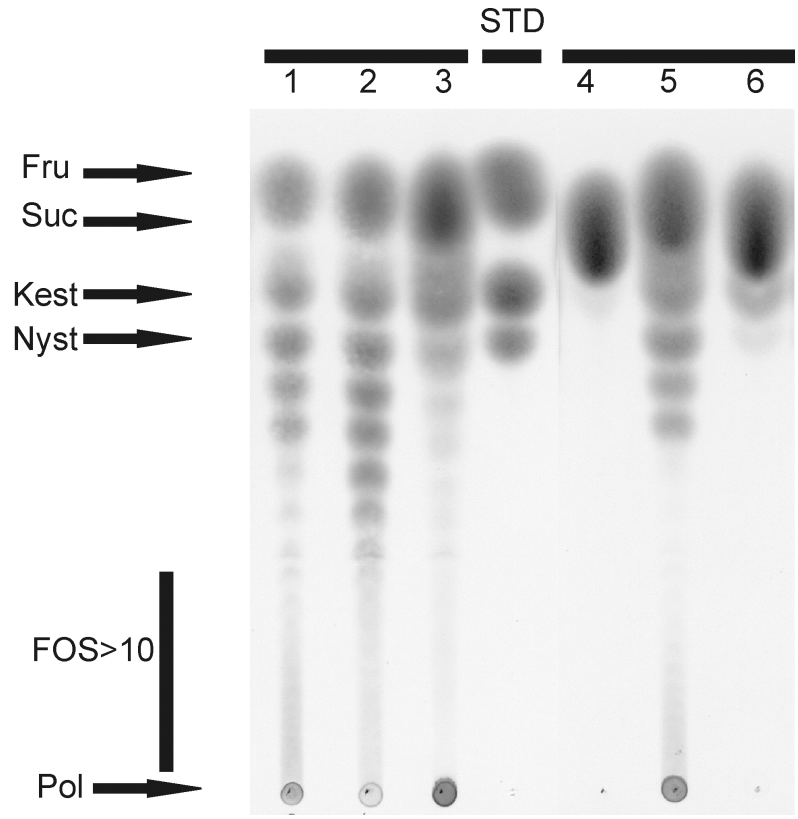

Figure 3. TLC analysis of the products synthesized by Inu WT, Lev WT and Inu mutants from sucrose (840 mM) at 37°C during a 24 h incubation period. The FTF enzymes were added as follows: Lane 1:W271N (2 U/ml); lane 2: Inu WT (2 U/ml); lane 3: Lev (2 U/ml); lane 4: R423H (0.016 U/ml); lane 5: R423K (0.31 U/ml); lane 6: W340N (0.25 U/ml). Reactions were performed in the standard reaction buffer. The plates were run once in butanol:ethanol:water (5:5:3) mixture. Fructose-containing sugars were specifically stained with urea spray [179]. Fru - fructose; Suc - sucrose; Kest - 1-kestose (GF2), Nyst - 1,1-nystose (GF3); FOS>10 – fructooligosaccharides with a degree of polymerization larger than 10; Pol – polymer.

4. Discussion

Structure-function relationships in bacterial FTF enzymes are not fully understood yet. Major questions still to be answered are: what structural features determine the type and size of polymers synthesized, and the ratio of FOS versus polymer produced.

Based on the available structural data, and an alignment of conserved regions in family GH68 enzymes (Figs. 1 and 2), eight residues appear to be involved in sucrose binding in the active site of these FTF enzymes. Inu residues D272, E523 and D424 were previously identified as catalytic nucleophile, acid/base catalyst and transition state stabilizer, respectively [123]. R541 (R360 in Bs SacB) was identified as a residue forming the acceptor binding site [25]. Three of the four remaining residues (W271, R423 and W340, but not E521) are completely conserved in family GH68 members and were selected as targets for site-directed mutagenesis.

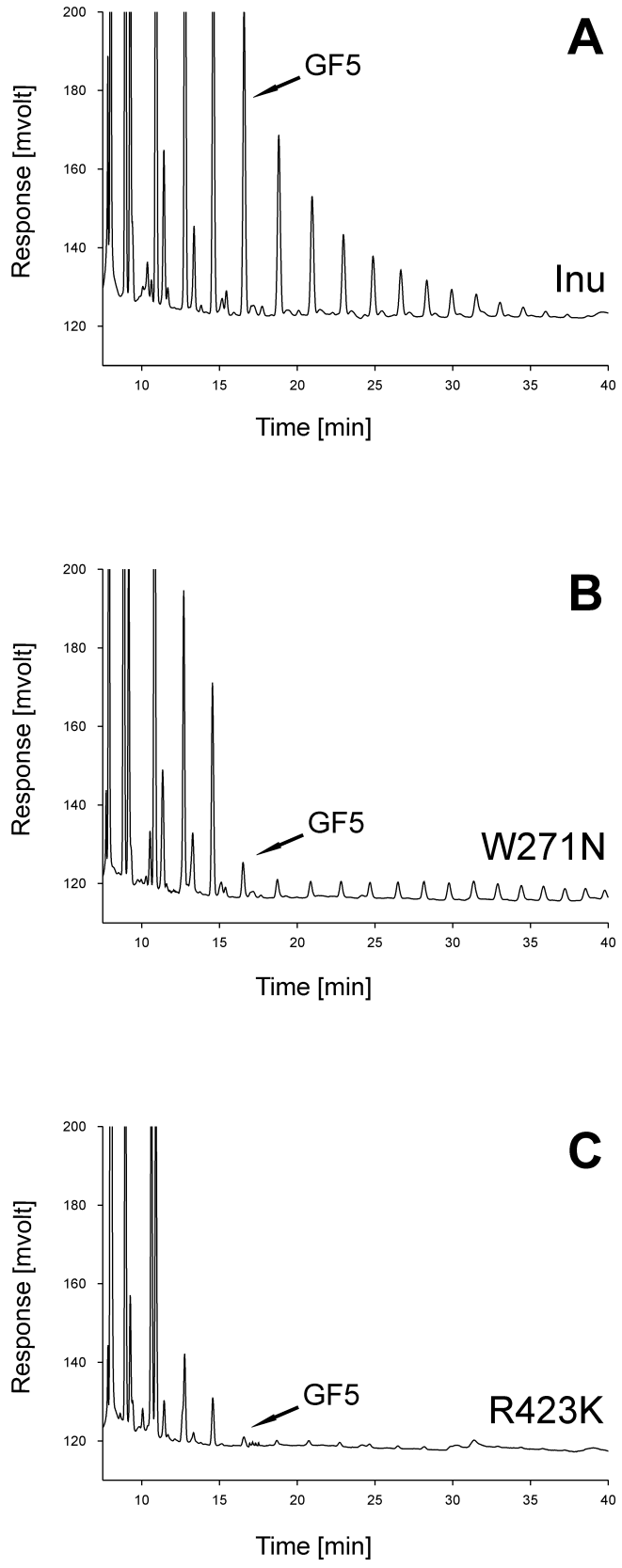

Figure 4. HP anion-exchange chromatography analysis of the products synthesized by Inu WT (A), and mutants W271N (B) and R423K (C), from sucrose (840 mM) at 37°C during a 24 h incubation period. The following amounts of enzymes were used: 2.2 U/ml of Inu, 2.6 U/ml of mutant W271N and 0.2 U/ml of mutant R423K.

The mutant Inu proteins displayed severe reductions in enzyme activities, showing that these residues make very important contributions, and are most likely involved in binding of the fructosyl unit of sucrose at the –1 subsite (W271, W340, R423) and the glucosyl unit of sucrose at the +1 subsite (R423) in the Inu active site (Fig. 1).

Inu **W271** (W85 in Bs SacB) is located at the -1 subsite and is conserved in the active site of all analyzed GH68 sequences (Figs. 1 and 2A). This residue is preceding the catalytic nucleophile (D272 in Inu and D86 in Bs SacB). As shown in the 3D structure of Bs SacB, W85 is located at the bottom of the active site and forms a hydrogen bond with the C6 hydroxyl group of the fructose unit [106]. Mutation W271N in *Lb. reuteri* Inu abolished the hydrogen bond and by this attenuated the interactions between enzyme and substrate, also strongly reducing enzyme activity. In family GH32 the equivalent position is occupied by an N residue [3].

Inu **W340** is conserved in GH68 family (Fig. 2B) and located very close to the fructose moiety of sucrose bound in the active site (Fig. 1). In family GH32 the equivalent position is occupied either by a tryptophan (1-EFH) or an phenylalanine residue (EI, invertase). Alberto *et al*. showed that F74 in *T. maritima* invertase, although not interacting with the substrate directly, is involved in sucrose binding, and borders the sucrose binding pocket [3]. F74 (invertase), W82 (1-EFH), and W163 (Bs SacB) are located at a similar position, although in 1-EFH the plane of the side chain is orientated differently [193]. Furthermore, W340 in Inu is adjacent to E270 – the equivalent of *Z. mobilis* E117 (Fig. 2B; see below). Aromatic residues are often involved in proper orientating polysaccharides in the acceptor-binding site of different enzymes [191]. Mutation W340N in *Lb. reuteri* Inu apparently disrupted the sucrose binding site, resulting in a very strong reduction in enzyme activity (Table 1).

Inu **R423** is part of the highly conserved "RDP motif" in GH68 family and it is also conserved in GH32 family members (Fig. 2C). This arginine residue precedes one of the catalytic residues (D424, transition state stabilizer) and has been reported to form a salt bridge with the third catalytic residue (the general acid/base catalyst) [104] while in Bs SacB it forms a salt bridge with the catalytic acid/base resdidue. Clearly it is a part of a complex network of interactions in the active site of these enzymes [106] and most likely influences the pKa of the respective acidic residue it ateracts with. R423 forms hydrogen bonds with both the fructose and glucose units of a bound sucrose at the –1 and +1 sugar binding subsites. Interestingly, enzymes belonging to families GH43 and GH62, that have a structurally related fold but hydrolyze different substrates, do not possess the equivalent R residue but only the catalytic D residue in the same position [3]. Nagem *et al*. reported that this R residue (R188 in EI; family GH32) participates in substrate binding, is important for recognition of the sugar ring and might be responsible for specificity of the enzyme toward fructopyranosyl residue [111].

The very low remaining activities of Inu mutants W340N and R423H confirmed that both residues are crucial for enzyme activity (Table 1). The introduced changes were severe: in mutant W340N the aromatic ring lining the sucrose-binding pocket was removed; in mutant R423H the bulky His residue probably disturbed the network of

interactions between the enzyme and both sucrose sugar moieties. Even in mutant R423K, introducing a less drastic change (residues of similar size and charge), the reduction in V_G was very significant (Table 1). The R423K mutation abolished the hydrogen bond with the glucose residue at the +1 subsite, but probably did not change interactions with fructose at the -1 subsite. In 24 h incubations a considerable transglycosylation activity remained and, compared to Inu WT, a clear increase was observed in the amount of inulin polymer synthesized as well as a shift in the range of FOS synthesized. Most striking was the virtually complete absence of FOS of DP7-9, in combination with a virtually unchanged amount of FOS of DP<7, and an increased synthesis of FOS/polymer of DP>10 (Fig. 3). HPAEC analysis of the reaction products provided clear evidence for synthesis of FOS up to DP6 only (Fig. 4C). Apparently, the larger size material observed on TLC (Fig. 3) represents FOS/polymer that was not visible in the HPAEC analysis.

Mutant Inu W271N clearly (Fig. 1 and 2A) synthesized FOS of DP10-20, present in similar amounts (Figs. 3 and 4B). Interestingly, both W271 and R423 are involved in binding of the fructosyl unit of sucrose at the -1 subsite in the active site (R423 also binds glucose in the +1 subsite). The mutant data clearly show that the interactions of both residues with the fructosyl residue not only are crucial for enzyme activity, but that changes in these interactions also strongly affect the size of the FOS synthesized and the amount of polymer produced. For unknown reasons, GH68 members strongly differ in the range and size of FOS/polymer synthesized. The FTF active site mutations analyzed in the present work targeted completely conserved residues in GH68, and therefore the experimental data presented here does not provide explanations for differences between GH68 members, and between *Lb. reuteri* 121 Inu and Lev. Rather, the mutations introduced may cause further structural changes in the layer of residues surrounding these completely conserved active site amino acids. Further 3D structural information is required to identify such putative changes.

The range of FOS synthesized by Inu (bacterial FTF, family GH68) resembles the reaction products of chicory 1-FFT (plant, family GH32) whereas the distribution of oligosaccharides made by Inu mutants W271N and R423K is very similar to products of globe thistle 1-FFT (plant, family GH32) [192]. A detailed analysis of the latter plant enzymes revealed that their differences in profiles of FOS synthesized are caused by differences in affinity of these enzymes for binding of short and long oligosaccharides. The high affinity of chicory 1-FFT for sucrose, fructose and kestose as substrate acceptors makes redistribution of fructose moieties from large to small fructans very likely (when the import and concentration of sucrose is high). The globe thistle 1-FFT enzyme has a lower affinity for sucrose and a high affinity for inulin, resulting in complete use of the 1-kestose synthesized by the second enzyme (1-SST) and further elongation of FOS molecules [192]. Based on this data we conclude that the Inu W271N and R423K mutations decreased affinity of the enzyme for sucrose and for short FOS, and increased the (relative) affinity for larger oligosaccharides and inulin.

The effect of mutations in the *B. subtilis* levansucrase acceptor substrate binding site on transglycosylation activity was studied by Chambert *et al*. [25]. Mutation of **R360**

(R541 in Inu; Fig. 2E), located in the +1 subsite (Fig. 1), into K, S or L, led to drastic changes in product profiles. Instead of accumulation of long chain levan, each of these mutants produced the trisaccharide kestose.

Yanase *et al*. reported that *Z. mobilis* levansucrase mutant **E117Q** (E339 in Inu; Fig 2B) exhibits significant changes in transglycosylation activity [200]. This *Z. mobilis* levansucrase showed higher polymerization ability than the WT protein. Moreover, the levan synthesized had a higher molecular mass than the polymer synthesized by WT protein. Residue E117 is located next to W118 (W340 in Inu) which contributes to formation of the –1 subsite. Its location close to the substrate-binding site may at least partly explain the strong effects of changes in this residue on enzyme activity and product specificity.

In conclusion, our data show that W271, W340 and R423 (*Lb. reuteri* Inu numbering) fulfill crucial roles in FTF enzymes, interacting with the bound sucrose at subsites –1 and +1. Changes in these residues strongly affect overall activity. Mutants W271N and R423K also display changes in their product specificities, especially in polymer versus fructooligosaccharide synthesis. The precise interactions between enzyme and fructose molecules bound to the active site thus are critical for the outcome of the FTF transglycosylation reaction. Our data show that the -1 subsite is not only important for substrate recognition and catalysis, but directly or indirectly also plays a role in determination of the size of products synthesized.

Chapter 6

Summary and concluding remarks

Lactic Acid Bacteria (LAB) are traditionally used for fermentative production of food and feed (e.g. wine, beer, bread, cheese, and yoghurt). LAB are Gram-positive bacteria and many possess the Generally Recognized as Safe (GRAS) status. LAB are known to produce extracellular polysaccharides (EPS) with multiple and often not fully understood physiological functions [39,53]. EPS may protect microbial cells against desiccation, phage attack, antibiotics and toxic compounds. Furthermore, EPS can be used as extracellular energy storage or may facilitate attachment to solid surfaces and biofilm formation (e.g. attachment to teeth, thereby causing formation of dental plague by *Streptococcus mutans*). Depending on their monosaccharide composition and biosynthetic pathways, EPS can be divided into two classes: (i) heteropolysaccharides, composed of different monosugars, synthesized from activated sugars by a complex, membrane associated, multiple enzyme system [39] and (ii) homopolysaccharides made up of one sugar type (e.g. fructans composed exclusively of fructose units) and synthesized from sucrose by a single, extracellular or cell-associated enzyme, known as sucrase (e.g. glucansucrase, fructansucrase) [50].

Enzymes of the sucrase type transfer a fructose (fructansucrases, fructosyltransferases, FTFs), or glucose (glucansucrases, glucosyltransferases, GTFs) unit from sucrose to the hydroxyl group of a water molecule (hydrolysis), or to a broad range of other sugar acceptor substrates. Depending on the acceptor substrate used, sucrases perform oligomerization (transfer of a monosaccharide to another sucrose molecule, or to fructo- or gluco-oligosaccharides) or polymerization (coupling of a monosaccharide to the growing fructan or glucan polymer chain). FTFs are divided into levansucrases, synthesizing levans with a majority of β(2-6) linked anhydrofructosyl residues and in some examples, where studied, considerably lower and varying amounts of β(2-1) linked branches, and inulosucrases making inulin with mainly β(2-1) linked anhydrofructosyl residues, and with some β(2-6) linked branches. Both types of enzymes thus may synthesize β(2-1) and β(2-6) linkages between anhydrofructose residues, but in a completely different ratio (**Chapter 1**).

Lactobacillus reuteri strain 121 (Lb121) is a representative of LAB and has the ability to produce large amounts of EPS, when grown on media containing sucrose. Of these polysaccharides about one-third is a reuteran, a highly branched glucan with α(1-4) and α(1-6) glycosidic linkages [90], and two-third is a levan. Only small amounts of inulin-type oligosaccharides were found [184]. Nevertheless, two FTF encoding (*ftf*) genes were detected in Lb121 and subsequently cloned and overexpressed in *Escherichia coli* [187,189]. Both enzymes were active and were further characterized with respect to products synthesized from sucrose. The first was a levansucrase (Lev) synthesizing a levan (Mr of 20,000 (97%) and 3-4 x 10^6 (3%); with β(2-6) linkages), and a small percentage of β(2-1) branches. The second FTF was an inulosucrase (Inu) synthesizing a high molecular mass inulin (Mr of 10^7; with β(2-1) linkages), and minor amounts of short fructooligosaccharides (FOS). Both enzymes were closely related at the amino acid sequence level (86 % similarity and 56 % identity, within 768 amino acids), raising

questions about structural features determining differences in substrate, reaction and product specificities.

Enzymes that hydrolyze a glycosidic bond are classified as glycoside hydrolases (GH) and are divided into about 100 different families (see http://afmb.cnrs-mrs.fr/CAZY/). Inu and Lev of Lb121 belong to family GH68 which includes almost 50 members from different origin (as for June 2005). They catalyze the hydrolysis of β-linked glycosidic bonds with the net retention of the configuration at the anomeric center. They use a Ping Pong type of mechanism, involving the formation and subsequent hydrolysis of a covalent enzyme-substrate intermediate [30]. The active site of β-retaining GH enzymes contains at least two catalytic amino acid residues: the nucleophile attacking at the sugar anomeric center to form a glycosyl-enzyme intermediate and a general acid/base catalyst, protonating the glycosidic oxygen in the first step and deprotonating a water molecule (or the hydroxyl group of an acceptor molecule) in the second step. Often a third residue, the transition state stabilizer, is present in the catalytic center of GH type of enzymes.

According to the generally accepted nomenclature, binding of a disaccharide in the active site of GH-type of enzymes occurs in the –1 and the +1 subsites. The numbering of the subsites depends on the position of the glycosidic bond hydrolyzed during the reaction (between position –1 and +1). Subsites are formed by amino acids localized close to each other and interacting with the same monosaccharide molecule. Based on the 3D structure of *B. subtilis* levansucrase (SacB) with sucrose bound to the active site, the amino acids constituting the –1 and the + 1 subsites have been identified [106]. When assuming that FTFs possess a domain interacting with the fructosyl acceptor substrate (i.e. a growing fructan chain), we propose that these enzymes contain additional subsites (+2, +3 etc.) that also may contribute strongly to overall enzyme substrate, reaction and product specificity (**Chapter 4**).

The major aims of this Ph.D. study were to identify and characterize structural features in the two FTFs of Lb121 that are relevant for the activities and product specificities of these enzymes. Here, we describe (i) the identification of the catalytic triad of the two Lb121 FTF enzymes, (ii) the location of the calcium binding site and the effects of calcium ions on the thermostability of Inu and Lev, (iii) a detailed characterization of the substrate, reaction and product specificities of these FTFs, and (iv) the identification of structural (amino acid) features within the enzymes important for determination of the specific ranges of fructooligosaccharides (FOS) and polymers synthesized.

Site-directed mutagenesis study of the catalytic triad in Lb. reuteri *121 FTF enzymes*

The catalytic residues of the two FTFs of Lb121 were identified by sequence alignments and mutagenesis studies (**Chapter 2**). An alignment of the amino acid sequences of Lb121 Inu, Lev, and the SacB of *B. subtilis*, of which the 3D structure had been solved [106], served to identify three conserved blocks of amino acids with strictly conserved carboxylates. The putative catalytic residues in Lev (Asp249, Asp404, Glu503) and Inu (Asp272, Asp424, Glu523) were changed into their amido counterparts (Asn or Gln) by site-directed mutagenesis, and the mutant proteins were produced in *E. coli*, purified and characterized. These mutations resulted in almost complete loss of activities, and in every case in more than 10,000 times reduced activities compared to the wild-type (WT) enzymes, indicating that these residues are essential for catalysis in these FTF enzymes. The highest reduction in activity was observed for the Asp249 (Lev) and Asp272 (Inu) residues located in the highly conserved family GH68 motif –V**WD**SW - (invariant residues in bold); these residues were identified as the catalytic nucleophile. Based on kinetic analysis of the Glu503Q (Lev) and Glu523Q (Inu), we concluded that Glu503 (and its equivalents) is the general acid/base catalyst. Finally, we conclude that Asp404 (Lev) and Asp424 (Inu) in the highly conserved family GH68 RDP motif constitute the third residue (transition state stabilizer) of the catalytic triad in the Lb121 FTF enzymes. Circular dichroism studies showed that only minor, if any, changes had occurred in the overall structure of the mutant proteins.

Mutational analysis of the role of calcium ions in Lb. reuteri *121 FTF enzymes*

Analysis of the *B. subtilis* SacB 3D structure provided evidence for the presence of a metal binding site, most likely binding calcium [106]. Chambert *et al*. demonstrated the importance of calcium ions for *in vitro* thermal refolding of *B. subtilis* levansucrase [26]. In **Chapter 3**, we analyzed the effect of calcium ions on the activity and thermostability of both Inu and Lev. Additionally, we made site-directed mutants in the possible calcium binding site of both enzymes and subsequently analyzed the affinity of these mutants for Ca^{2+} binding. The results showed that Ca^{2+} ions play a major structural role in the Inu and Lev proteins, strongly affecting their activities in relation to their temperature optima as well their thermostability [122].

In the presence of extra Ca^{2+} ions, the Inu and Lev WT enzymes were considerably more active at higher temperatures. The presence of calcium thus appeared to be essential to prevent inactivation of both enzymes at high temperatures. In order to analyze the effect of Ca^{2+} ions on thermostability, both enzymes were incubated at different temperatures in the presence and absence of Ca^{2+}, followed by analysis of the remaining activity at 37°C. A drastic loss of activity was observed for Inu after preincubation of the enzyme at 45-50°C for 30 min. The presence of 1 mM $CaCl_2$, however, provided protection to the enzyme. Thermostability of Lev also depended on the presence of Ca^{2+}

ions, although differing from what was observed for Inu and calcium. Lev also showed a clear reduction in activity at higher temperatures but in contrast to Inu, it did not suffer irreversible inactivation [122].

Analysis of site-directed mutants in the putative calcium-binding sites of Inu and Lev revealed that Asp520 (Inu) and Asp500 (Lev) are crucial for binding of calcium ions. The mutant proteins Asp520Asn, Ala and Asp500Asn, Ala displayed a strong reduction in activity over a broad temperature range. The stimulatory effects of the addition of extra Ca^{2+} ions on enzyme activities versus temperature were much smaller in the mutant enzymes (virtually abolished in the Asp to Ala mutants) than in the WT enzymes. The Asp to Asn mutant enzymes were still able to bind Ca^{2+} ions, although very weakly. Inu mutant Asp520Asn displayed a severe reduction in Ca^{2+} affinity (approximately 1600-fold reduction in K'_d value) while the affinity for Ca^{2+} of the Lev mutant Asp500Asn was reduced only approximately 35-fold.

The data thus show that Ca^{2+} ions have an important structural role in both the Inu and Lev enzymes. Both proteins possess high-affinity calcium binding sites constituted, amongst others, by the Asp residue located in the **D**EI**ER** motif strongly conserved in family GH68. This Asp residue is not conserved in FTF enzymes of Gram-negative bacteria that probably do not bind Ca^{2+} ions. Recently, the first 3D structure of an FTF enzyme from *Gluconacetobacter diazotrophicus*, a Gram-negative bacterium, was published [104], confirming our predictions.

Transglycosylation reactions catalyzed by Lb. reuteri *121 FTF enzymes*

A detailed analysis of the transglycosylation reactions catalyzed by Inu and Lev revealed similarities but also major differences between these two enzymes (**Chapter 4**). The Lev transglycosylation activity mainly resulted in synthesis of a large molecular weight levan polymer. Inu, on the contrary, synthesized a broad range of FOS and relatively little of the inulin polymer. Inu synthesized FOS according to a non-processive reaction, in which the growing fructan chain is released after (virtually) each transfer of a fructosyl unit. The products synthesized at the early stages of the reaction thus serve as acceptor substrates in further elongation steps. The characteristic TLC ladder-like profile of inulin-type FOS suggested that the enzyme has a higher affinity for shorter oligosaccharides than for longer ones. The Lev enzyme, on the contrary, synthesized only small amounts of short oligosaccharides and much larger amounts of polymer, compared to Inu. These results suggested that Lev catalyzed a processive type of reaction, i.e. the growing levan polymer chain remained attached to the protein and is only released after the polymer reaches a certain length. Because the eight amino acid residues present in the active sites of both enzymes are almost the same, we propose that the two enzymes most likely differ in further acceptor substrate binding subsites (i.e. +2, +3 subsites), i.e. with different residues involved, resulting in a different overall architecture.

Inu and Lev also used raffinose and sucrose-related inulin-type of oligosaccharides with a degree of polymerization of 3 to 5 (1-kestose, 1,1-nystose, 1,1,1-kestopentaose) as

substrates. TLC analysis of the reaction products showed that Inu converted these substrates much more efficiently than Lev. The main activity of both enzymes was the production of FOS that was one fructosyl residue smaller or larger than the substrates used. These data suggested that Inu and Lev performed a disproportionation type of reaction with these larger substrates. Interestingly, both FTF enzymes were unable to synthesize polymer from these larger oligosaccharides as sole substrate. With Inu this had been observed already with sucrose (see above) and explained by weak ability of subsites +2, +3 etc. to bind the growing fructan chain. Apparently, the Lev catalyzed polymerization reaction requires disaccharide (sucrose, binding at subsites -1 and +1) as a donor substrate. Polymer synthesis from larger size FOS donor substrates most likely fails because subsites +2, +3, and further remain occupied by the growing fructan acceptor molecule (processive type of reaction in case of Lev). Using larger FOS as fructosyl donors necessitates release of the acceptor molecule after each transglycosylation step, thus resulting in a non-processive type of reaction (oligomerisation), also in case of Lev.

Polymer versus fructooligosaccharide synthesis in Lb. reuteri *121 FTF enzymes*

The *Lb. reuteri* 121 FTF enzymes strongly differ in products synthesized via the polymerization and oligomerization reactions. **Chapter 5** aimed to identify structural (amino acid) features that contribute to these differences in product specificities. Three different residues in the Lb121 Inu enzyme were selected for site-directed mutagenesis, and the mutant proteins were subsequently characterized biochemically. Based on the 3D structure of the *B. subtilis* SacB protein, Trp340 of Inu residue was identified as bordering the sucrose-binding pocket [106]. Inu mutant Trp340Asn was virtually inactive (0.5% of Inu WT activity), confirming the essential role of this residue in the FTF active site. Also Inu mutant Arg423His was virtually inactive (0.3% of Inu WT activity), most likely due to disruption of the fine network of interactions created by the similar Arg residue around the subsites –1 and +1 in the *B. subtilis* SacB protein [106].

Inu mutant Arg423Lys was less strongly affected in activity and displayed an altered FOS product pattern from sucrose. Compared to Inu WT, the mutant Arg423Lys enzyme produced a much lower amount of oligosaccharides and significantly more polymer. Similar results were obtained with Inu mutant Trp271Asn. Interestingly, both these residues (Arg423 and Trp271) are located at the bottom of the active site and constitute the -1 subsite. Based on these preliminary data it was concluded that the -1 subsite is not only important for substrate recognition but also contributes to determination of the size of the products synthesized.

In the work presented here, we identified the catalytic triads of the two FTFs of *Lb. reuteri* 121. Such an analysis of mutant enzyme properties, combined with a determination of the resulting catalytic activity and the kinetic properties for one and the same FTF enzyme, had not been published before.

Asp500 (Lev) and Asp520 (Inu) residues have essential structural role in the calcium-binding pocket of these proteins. Mutations in these Asp residues affect both

enzyme activities versus temperature and protein thermostability. The residues constituting the Ca^{2+} binding site are completely conserved in most family GH68 enzymes from Gram-positive bacteria, suggesting that the stabilizing function of calcium ions is a general property of these proteins. The calcium-binding site appears to be absent in family GH68 enzymes of Gram-negative bacteria.

The two Lb121 FTF enzymes, although very similar at the amino acid sequence level (86 % similarity and 56 % identity, within 768 amino acids), catalyzed significantly different transglycosylation reaction. Inu synthesized much more FOS and Lev produced mainly levan polymer. This was interpreted as indicating hitherto unidentified structural differences in acceptor substrate binding subsites +2, +3, etc.

Finally, it was shown that changes in amino acid residues involved in binding of the fructose unit of sucrose in the Inu and Lev active site contribute to determination of the size of fructans made. Elucidation of 3D structures of the *Lb. reuteri* 121 inulosucrase and levansucrase proteins with sucrose and / or a larger size acceptor substrate molecules bound in the active site may help in understanding why such similar enzymes differ so strongly with regards to the amounts of oligosaccharides versus polymer synthesized, and the type of glycosidic linkage made.

Chapter 7

Samenvatting en conclusies

Melkzuurbacteriën worden van oudsher gebruikt bij fermentatieprocessen in de voedingsmiddelen en veevoeder industrie, zoals bij de bereiding van wijn, bier, brood, kaas en yoghurt. Het zijn Gram-positieve bacteriën en vele soorten hebben een GRAS (Generally recognised as Safe of tewel algemeen aanvaard als veilig) status. Melkzuurbacteriën kunnen extracellulaire polysacharides (EPS) produceren met verschillende, vaak nog slecht begrepen fysiologische functies [39,53]. EPS kan de cellen resistent maken tegen uitdroging, bacteriofagen, antibiotica en toxische verbindingen. Daarnaast kunnen melkzuurbacteriën de EPS gebruiken als extracellulaire energie voorraad, voor aanhechting op een vaste ondergrond, bij de vorming van biofilms resulterend in vorming van tandplak door bijvoorbeeld *Streptococcus mutans*. EPS kunnen op basis van de hun samenstelling en de biosynthese route onderverdeeld worden in twee groepen, te weten (i) heteropolysacharides die opgebouwd zijn uit verschillende soorten suiker eenheden en welke door een complex, membraan gebonden, enzymsysteem gemaakt worden uit geactiveerde suikers, en (ii) homopolysacharides die bestaan uit één type suiker, zoals bijvoorbeeld fructanen en glucanen die opgebouwd zijn uit enkel en alleen fructose of glucose eenheden, en die gemaakt worden uit sucrose (tafelsuiker) door een enkelvoudig, extracellulair of aan de cel gebonden enzym, een zogenaamde sucrase (glucan- en fructansucrases) [50].

Enzymen van het sucrase type zetten of de fructose (fructansucrases, fructosyltransferases, FTFs) of de glucose (glucansucrases, glucosyltransferases, GTFs) eenheid van sucrose over op een hydroxyl groep van een watermolecuul (de hydrolyse reactie), of op een variatie aan andere suiker acceptoren (de acceptor reactie). Afhankelijk van het acceptor substraat dat wordt gebruikt katalyseren sucrase enzymen een oligomerizatie reactie (overdracht van een enkel suiker molecuul op een ander sucrose of fructo/gluco-oligosacharide molecuul), of een polymerizatie reactie (koppeling van een enkel suiker molecuul op de groeiende fructan of glucan keten). FTFs kunnen onderverdeeld worden in levan- en inulosucrases. Levansucrases maken levan polymeren die bestaan uit anhydrofructosyl eenheden met voornamelijk β(2-6) en enkele β(2-1) bindingen. Inulosucrases maken inuline polymeren met voornamelijk β(2-1) en enkele β(2-6) gebonden anhydrofructosyl eenheden. Zowel levan- als inulosucrases maken dus een polysacharide met zowel β(2-1) als β(2-6) gebonden anhydrofructosyl eenheden maar in een totaal andere verhouding (zie ook **Hoofdstuk 1**).

Lactobacillus reuteri stam 121 (Lb 121) is een melkzuurbacterie en produceert een grote hoeveelheid EPS uit sucrose. Van dit EPS is ongeveer eenderde deel reuteran, een sterk vertakt glucan met zowel α(1-4) als ook α(1-6) glycosidische bindingen [90], en tweederde deel is levan. Ook werd een kleine hoeveelheid inulinachtige oligosachariden gevonden [5]. Twee *ftf* genen coderend voor twee verschillende FTF enzymen werden gevonden in het genoom van Lb121. Deze *ftf* genen zijn gekloneerd en tot overexpressie gebracht in *Escherichia coli* [187,189]. Beide enzymen waren actief en zijn nader gekarakteriseerd voor wat betreft de producten die ze uit sucrose maken. Het eerste

enzym was een levansucrase (Lev) dat een levan produceerde (Mr van 20.000 (97%) en 3-4 x 10^6 (3%)) met voornamelijk β(2-6) bindingen, en een klein gedeelte β(2-1) bindingen. Het tweede FTF enzym was een inulosucrase (Inu) dat een hoog molecuul gewicht inuline produceerde (Mr van 10^7) met voornamelijk β(2-1) bindingen, plus een kleine hoeveelheid fructo-oligosachariden met korte ketenlengtes. Beide enzymen vertoonden een hoge mate van gelijkenis op aminozuur niveau (86% gelijke en 56% identieke aminozuren over een lengte van 768 aminozuur residuen). Dit gaf aanleiding tot vragen over welke onderdelen van de structuur van de enzymen verantwoordelijk is voor verschillen in substraat, reactie en product specificiteiten.

Enzymen die een glycosidische binding hydrolyseren worden aangeduid als glycoside hydrolasen (GH) en zijn onderverdeeld in ongeveer 100 verschillende families (zie http://afmb.cnrs-mrs.fr/CAZY/). Inu en Lev van Lb 121 zijn leden van familie GH68 welke bestaat uit bijna 50 leden van allerlei oorsprong (stand van zaken in Juni 2005). Dergelijke enzymen katalyseren de hydrolyse van β-gebonden glycosidische bindingen met behoud van de configuratie van het anomerische centrum. Ze gebruiken een Ping Pong reactie mechanisme met vorming en vervolgens hydrolyse van een covalent gebonden enzym-substraat intermediair [30]. Het actieve centrum van β-behoudende GH enzymen bevat ten minste twee katalytische aminozuur residuen: de katalytische nucleofiel die het anomere centrum van het suiker molecuul aanvalt en vervolgens betrokken is bij de vorming van het glycosyl-enzym intermediair, en de algemene zuur/base katalysator die een proton afstaat aan de glycosidische zuurstof in een eerste stap van de reactie en vervolgens in de tweede stap een proton van een water molecuul afhaalt (of van een hydroxyl groep van een suiker acceptor molecuul). Vaak is ook een derde aminozuur residue, de transitie toestand stabilisator, aanwezig in het katalytische centrum van GH type enzymen.

De binding van een disacharide in het actieve centrum van GH enzymen vindt, op basis van een algemeen aanvaarde naamgeving, plaats in de -1 en +1 subsite. De nummering van de subsites hangt af van de positie van de glycosidische binding die tijdens de enzym reactie gehydrolyseerd wordt (tussen de -1 en +1 positie). Subsites worden gevormd door aminozuur residuen die dichtbij elkaar gelokaliseerd zijn en die een interactie met hetzelfde suiker molecuul hebben. Met behulp van een 3D structuur van het *Bacillus subtilis* levansucrase (SacB), met een sucrose gebonden in het actieve centrum, zijn de aminozuur residuen die de -1 en +1 subsite vormen geïdentificeerd [106]. Aannemende dat FTFs een domein hebben dat een interactie met de fructosyl eenheid van het acceptor substraat (dat is de groeiende fructan keten) stellen wij voor dat deze enzymen extra subsites (+2, +3, etc) hebben die ook een duidelijke bijdrage leveren aan de algemene substraat, reactie en product specificiteit (**zie Hoofdstuk 4**).

De belangrijkste doelen van dit promotie onderzoek waren het identificeren en bestuderen van de structuur/functie relaties in de twee FTF enzymen van Lb121, met

name gericht op de specifieke activiteit en product specificiteit. Het onderzoek resulteerde in de volgende, in dit proefschrift beschreven, resultaten: (i) identificatie van de 3 katalytische aminozuur residuen van beide FTF enzymen van Lb 121; (ii) lokalisatie van de calcium bindingsplaats in Inu en Lev, en analyse van het effect dat calcium ionen hebben op de thermostabiliteit van Inu en Lev; (iii) een gedetailleerde karakterisatie van de substraat, reactie en product specificiteit van de FTF enzymen en (iv) de identificatie van de aminozuur residuen in de enzymen die een belangrijke rol spelen bij de vorming van de specifieke serie van fructo-oligosacharides (FOS) en polymeren die door de twee enzymen gemaakt worden.

Mutagenese van de katalytische residuen van Lb. reuteri *121 FTF enzymen*

De katalytische residuen in de twee Lb 121 FTF enzymen zijn geïdentificeerd op basis van aminozuur volgorde vergelijkingen, gecombineerd met plaatsgerichte mutagenese (**Hoofdstuk 2**; [123]). De aminozuur volgorde van het Lb121 Inu en Lev zijn vergeleken met die van SacB van *B. subtilis*, waarvan recentelijk ook de 3D structuur opgehelderd is [106]. Op basis van deze vergelijking zijn drie geconserveerde regio's van aminozuur residuen geïdentificeerd waarin strikt geconserveerde Asp en Glu residuen aanwezig zijn. De waarschijnlijke katalytische residuen in Lev (Asp249, Asp404, Glu503) en in Inu (Asp272, Asp424, Glu523) zijn vervolgens m.b.v. plaatsgerichte mutagenese veranderd in Asn of Gln residuen, waarna de mutante enzymen in *E. coli* geproduceerd zijn, en na zuivering biochemisch gekarakteriseerd. Deze mutaties resulteerden in een vrijwel volledig verdwijnen van enzym activiteit: in alle gevallen werd waargenomen dat de enzymactiviteit meer dan 10.000 keer gereduceerd werd vergeleken met het wild type (WT) enzym. Deze aminozuur residuen zijn dus essentieel voor katalyse in deze FTF enzymen. De grootste reductie van de activiteit werd gevonden bij verandering van de residuen Asp249 (Lev) en Asp272 (Inu), die zich bevinden in het binnen de familie GH68 zeer sterk geconserveerde motief –V**WD**SW- (met de absoluut geconserveerde residuen in vet). Deze Asp residuen zijn geïdentificeerd als de katalytische nucleofiel. Op basis van een analyse van de enzym kinetiek van de mutanten Glu503Q (Lev) en Glu523Q (Inu) is geconcludeerd dat Glu503 en Glu523 de algemene zuur/base katalysator moet zijn. Ook is geconcludeerd dat Asp404 (Lev) en Asp424 (Inu), welke onderdeel uitmaken van het zeer geconserveerd RDP motief in familie GH68 enzymen, het derde residu, te weten de transitie toestand stabilisator, van de katalytische triade van Lb121 FTF enzymen moet zijn. Analyse van de structuur van de mutante enzymen met behulp van circulair dichroisme studies gaf aan dat slechts geringe structurele veranderingen waren opgetreden.

Analyse van de rol van calcium ionen in Lb. reuteri *121 FTF enzymen*

Bestudering van de 3D structuur van het *B. subtilis* SacB eiwit gaf aan dat in dit enzym een bindingsplaats voor metaal ionen, zeer waarschijnlijk calcium, aanwezig is

[106]. Chambert *et al.* hadden al eerder het belang van calcium ionen aangetoond bij de thermische terugvouwing van het *B. subtilis* levansucrase [26]. In **Hoofdstuk 3** worden de effecten van calcium ionen op de activiteit en thermostabiliteit van Inu en Lev beschreven. Ook worden de eigenschappen van plaatsgerichte mutanten, met veranderingen in de aminozuur residuen die mogelijkerwijs bij calcium binding betrokken zijn, beschreven met name ten aanzien van de affiniteit voor calcium binding. De resultaten geven aan dat calcium ionen een zeer belangrijke structurele rol spelen in de Inu en Lev enzymen, met een sterke invloed op de activiteiten van beide enzymen in relatie tot hun temperatuur optima, en op hun thermostabiliteit.

In de aanwezigheid van extra calcium ionen zijn zowel het Inu als Lev enzym beduidend meer actief bij hogere temperaturen. De aanwezigheid van calcium lijkt dus essentieel te zijn om inactivatie van beide enzymen bij hogere temperaturen te voorkomen. Om het effect van calcium ionen op de thermostabiliteit beter te bestuderen werden beide enzymen enige tijd geïncubeerd bij verschillende temperaturen in de aan- of afwezigheid van calcium. Hierna werd de resterende activiteit van beide enzymen gemeten bij 37^{o}C. Een zeer groot verlies aan activiteit werd gevonden bij het Inu enzym nadat dit enzym gedurende 30 min eerst bij 45-50^{o}C was geïncubeerd. Toevoeging van 1 mM $CaCl_2$ gaf echter bescherming aan het enzym. De thermostabiliteit van het Lev enzym was ook afhankelijk van de aanwezigheid van calcium ionen. Lev liet ook een sterke reductie van de activiteit zien bij hogere temperaturen. In tegenstelling tot Inu was de inactivatie van Lev niet onherstelbaar [122].

Analyse van mutante Lev en Inu enzymen, met plaatsgericht veranderingen in de aminozuur residuen die de mogelijk calcium bindingsplaat vormen, liet zien dat Asp520 (Inu) en Asp500(Lev) cruciaal zijn voor binding van calcium ionen. Mutante enzymen waarin het Asp residu op positie 520 in het geval van Inu en positie 500 in het geval van Lev veranderd waren in een Asn of Ala lieten een sterke reductie van de activiteit over een breed temperatuur bereik zien. Het stimulerende effect dat extra calcium normaal heeft op de enzym activiteit van Inu of Lev in relatie tot de temperatuur was indien de hierboven genoemde mutante enzymen werden gebruikt veel kleiner. Bij de verandering van Asp naar Ala was dit effect zelfs vrijwel helemaal teniet gedaan. De Asp naar Asn mutante enzymen waren nog steeds in staat calcium te binden, zij het dat de binding zeer zwak was geworden. De Inu mutant Asp520Asn liet een zeer sterke reductie zien in de affiniteit voor calcium; een 1600-voudige reductie van de K'_d waarde werd gemeten. Dit terwijl de affiniteit van de Lev mutant Asp500Asn slechts een 35-voudige reductie van de affiniteit te zien gaf.

Deze resultaten laten zien dat calcium ionen een belangrijke structurele rol spelen in de Inu en Lev enzymen. Beide eiwitten hebben een hoge-affiniteit calcium bindingsplaats welke onder meer gevormd wordt door het Asp residue aanwezig in het sterk geconserveerde motief **D**EI**ER** in familie GH68. Het betreffende Asp residu is overigens niet geconserveerd in FTF enzymen die beschreven zijn voor Gram-negatieve bacteriën. Op basis hiervan voorspelden we dat FTF enzymen van Gram-negatieve bacteriën hoogstwaarschijnlijk geen calcium ionen binden. Recent is de eerste 3D structuur van een

FTF eiwit van een Gram-negatiëve bacterie, te weten *Gluconacetobacter diazotrophicus*, beschreven [104] en dit bevestigde de hiervoor beschreven voorspelling.

Transglycosyleringsreacties gekatalyseerd door Lb. reuteri *121 FTF enzymen*

Een gedetailleerde bestudering van de transglycosyleringsreacties gekatalyseerd door Inu en Lev liet duidelijke overeenkomsten maar ook verschillen tussen beide enzymen zien (**Hoofdstuk 4**). De transglycosyleringsreactie van Lev resulteerde vooral in synthese van een levan polymeer met een hoog molecuul gewicht. Inu, daarentegen, maakte vooral een brede reeks aan FOS en relatief weinig inuline polymeer. Inu maakte de FOS via een "non-processive" reactie, waarbij de groeiende fructan keten na iedere verlenging met een fructose eenheid eerst weer vrij komt van het enzym. De producten die in het begin van de reactie gemaakt worden dienen vervolgens weer als acceptor substraten bij een volgende transfer reactie. Op dunne laag chromatografie gaf dit een karakteristiek ladder-achtig profiel van inuline-type FOS. Het enzym heeft vermoedelijk een hogere affiniteit voor kortere dan voor langere oligosachariden. Het Lev enzym daarentegen maakte slechts een kleine hoeveelheid van de kortere oligosachariden en veel meer van het levan polymeer. Deze resultaten geven aan dat Lev een "processive" reactie katalyseert, waarbij de groeiende levan polymeer keten aan het enzym vast blijft zitten en pas losgelaten wordt nadat het polymeer een zekere ketenlengte bereikt heeft. Omdat in beide enzymen vrijwel dezelfde aminozuur residuen aanwezig zijn in het actieve centrum, stellen we voor dat de twee FTF enzymen verschillend zijn in de organisatie en samenstelling van de naast de +1 subsite gelegen acceptor substraat bindingsplaatsen, dat wil zeggen dat er verschillen zijn in de aminozuur residuen die deze subsites vormen waardoor deze een verschillende ruimtelijke architectuur hebben.

Inu en Lev kunnen beiden ook raffinose en aan sucrose verwante inuline-achtige oligosachariden (1-kestose, 1,1-nystose en 1,1,1-kestopentaose, met een polymerizatiegraad van respectievelijk 3 tot 5 suiker eenheden) gebruiken als substraten. Analyse van de reactie producten met behulp van dunne laag chromatografie liet zien dat Inu deze substraten meer effectief omzette dan Lev. De belangrijkste activiteit van beide enzymen was productie van FOS dat een fructose residu kleiner of groter was dan het substraat dat gebruikt werd. Deze resultaten laten zien dat Inu en Lev een disproportioneringsreactie uitvoeren met deze grotere substraten. Het was opvallend dat beide enzymen niet in staat waren polymeer te synthetiseren uit deze substraten. Een soortgelijke waarneming was al gedaan voor Inu met sucrose als substraat, zoals hierboven al uitgelegd, hetgeen werd verklaard door een zwakke bindingsaffiniteit van de subsites +2, +3 enz van het Inu enzym met de groeiende fructan keten. Blijkbaar kan de Lev enzym gekatalyseerde polymerizatie reactie enkel een disacharide (zoals sucrose, welke bindt aan subsites -1 en +1) gebruiken als donor substraat. Lev is niet in staat tot polymeer synthese vanuit grotere FOS donor substraten zoals 1-kestose en 1,1-nystose omdat subsites +2, +3 en verder bezet blijven door de groeiende fructan keten (hetgeen

kenmerkend is voor een "processive" type reactie). Om langere FOS als fructosyl donoren te kunnen gebruiken moet de groeiende fructan acceptor keten los gelaten worden na iedere transglycosyleringsstap. Dit is karakteristiek voor een "non-processive" type reactie (oligomerizatie) zoals het geval is bij het Lev enzym.

Polymeer versus fructo-oligosacharide synthese door Lb. reuteri *121 FTF enzymen*

De twee *Lb. reuteri* 121 FTF enzymen verschillen sterk in producten van de polymerisatie en oligomerisatie reacties. In **Hoofdstuk 5** hebben we, op basis van de 3D structuur van het *B. subtilis* SacB enzym, drie volledig geconserveerde residuen geïdentificeerd die betrokken zijn bij substraat binding in het actieve centrum. Deze 3 aminozuren zijn vermoedelijk ook in het Lb121 Inu enzym betrokken bij sucrose binding. Deze 3 aminozuren zijn geselecteerd voor mutagenese. Deze mutante enzymen werden vervolgens biochemisch gekarakteriseerd. Het Trp340 residu van Inu werd geïdentificeerd als een belangrijk aminozuur grenzend aan de sucrose bindingsplaats [106]. De Inu mutant Trp340Asn bleek vrijwel inactief te zijn (0,5% van de activiteit van het wild type enzym), waarmee een duidelijk aanwijzing gevonden werd voor de essentiële rol van dit residu in het actieve centrum van dit FTF enzym. Ook de Inu mutant Arg423His was vrijwel inactief (0,3% van de wild type activiteit). Het vergelijkbare Arg residu in het *B. subtilis* SacB enzym vormt een fijnmazig netwerk van interacties rondom de -1 en +1 subsites [106]. Inu mutant Arg423Lys was minder sterk gereduceerd in activiteit, maar had een sterk veranderd product profiel. De Arg 423Lys mutant produceerde veel minder oligosachariden en beduidend meer polymeer dan wild type Inu. Vergelijkbare resultaten werden gevonden voor Inu mutant Trp271Asn. De Arg423 en Trp271 residuen bevinden zich beiden op de bodem van het actieve centrum van Inu en vormen de -1 subsite. Op basis van deze voorlopige resultaten werd geconcludeerd dat de -1 subsite niet alleen van belang is voor de herkenning van het substraat maar ook mede bepalend is voor de lengte van de producten gemaakt uit het substraat sucrose.

In het voorliggende proefschrift wordt de identificatie van de 3 aminozuur residuen betrokken bij katalyse in de twee FTF enzymen van *Lb. reuteri* 121 beschreven. Een dergelijke analyse van de eigenschappen van alledrie de mutante FTF enzymen, gecombineerd met een bepaling van de katalytische activiteiten en de kinetische eigenschappen, was nog niet eerder gepubliceerd voor een en hetzelfde FTF enzym.

De Asp500 (Lev) en Asp520 (Inu) aminozuur residuen bleken een essentiële structurele rol te hebben in de calcium bindingsplaats van beide enzymen. Mutagenese van deze Asp residuen heeft een duidelijk effect op de activiteit en stabiliteit van beide enzymen in relatie tot temperatuur. De aminozuur residuen die de calcium bindingsplaats vormen zijn absoluut geconserveerd in de meeste familie GH68 enzymen van Gram-positieve bacteriën, hetgeen suggereert dat de stabiliserende werking van calcium een algemene eigenschap is in deze groep van enzymen. De calcium bindingsplaats blijkt afwezig te zijn in familie GH68 enzymen van Gram-negatieve bacteriën.

De twee Lb 121 FTF enzymen zijn in zeer hoge mate aan elkaar gelijk qua aminozuur volgorde (86% gelijke en 56% identieke aminozuren over een lengte van 768 aminozuur residuen) maar verschillen sterk in producten van de transglycosyleringsreactie. Inu maakt veel meer FOS en Lev maakt voornamelijk levan polymeer. Op basis hiervan speculeerden we dat er structurele verschillen zijn in de (nog niet geïdentificeerde) acceptor bindingsplaatsen +2, +3 enz. van beide enzymen.

Ook werd waargenomen dat veranderingen in aminozuur residuen die hoogstwaarschijnlijk betrokken zijn bij de binding van de fructose eenheid in sucrose in het actieve centrum van Inu resulteren in variaties in de ketenlengte van het fructan dat gemaakt wordt. Opheldering van de 3D structuren van de *Lb. reuteri* 121 inulosucrase en levansucrase eiwitten, bij voorkeur met een sucrose en/of langere acceptor substraat moleculen gebonden in het actieve centrum, zou duidelijk kunnen maken waarom dergelijke zo op elkaar lijkende enzymen zo verschillen in de hoeveelheden oligosachariden en polymeer die ze synthetiseren, en in het type glycosidische binding in het product.

Chapter 8

Podsumowanie

Bakterie mlekowe (BM) są od wieków używane do fermentacji żywności i pasz dla zwierząt (np. w produkcji wina, piwa, chleba, serów żółtych i jogurtów). Do BM należą tylko bakterie Gram-dodatnie, z których wiele posiada status GRAS (Generally Recognized As Safe), oznaczający, że mogą one być stosowane przy produkcji żywności. BM produkują duże ilości polisacharydów (wielocukrów) i wydzielają je do medium, w którym żyją. Takie cząsteczki cukrowe nazywamy egzopolisacharydami (EPS). Cząsteczki EPS, otaczające komórki bakteryjne, chronią je przed wysychaniem, przed działaniem antybiotyków i toksyn oraz przed atakiem bakteriofagów. Dodatkowo EPS mogą być używane jako zewnątrzkomórkowy magazyn energii lub mogą pomagać w trwałym zamocowaniu bakterii do gładkich powierzchni, np. do zębów w tzw. kamieniu nazębnym. W zależności od sposobu ich syntezy oraz rodzaju monocukrów, z których są zbudowane, EPS możemy podzielić na heteropolisacharydy składające sie z różnych cząsteczek cukrów prostych i syntetyzowane przez kompleks enzymów związanych z błoną komórkową bakterii oraz homopolisacharydy, zbudowane w całości z jednego rodzaju cukrów prostych i syntetyzowane przez jeden zewnątrzkomórkowy enzym zakotwiczony do ściany komórkowej komórki bakteryjnej. Substratem do syntezy heteropolisacharydów są aktywowane cząsteczki cukrów prostych, a homopolisacharydy syntetyzowane są z sacharozy (czyli zwykłego cukru stołowego) przez enzymy zwane cukrazami. Sacharoza jest dwucukrem zbudowanym z jednej cząsteczki glukozy i jednej cząsteczki fruktozy połączonych wiązaniem glikozydowym Właśnie badaniami nad takimi enzymami (cukrazami) zajmowałem się podczas swoich studiów doktoranckich.

Cukrazy katalizują hydrolizę sacharozy do monocukrów, które mogą być uwolnione do medium, gdzie przebiega reakcja (hydroliza). W przypadku hydrolizy cząsteczki wody możemy uznać za akceptory dla uwalnianych monocukrów. Poza wodą cukrazy mogą używać wielu innych akceptorów, jeśli są one dostępne. I tak użycie jako akceptora krótkiego łańcucha cukrowego (oligosacharydu) nazwiemy oligomeryzacją, a użycie cząsteczki polimeru cukrowego – polimeryzacją. Zarówno oligomeryzacja jak i polimeryzacja prowadzą do powstania cząsteczek akceptora przedłużonego o jedną resztę cukrową na każdy cykl reakcji. Fruktancukrazy (FTF-y) katalizują transfer fruktozy do cząsteczki akceptora uwalniając przy tym cząsteczkę glukozy do medium reakcyjnego, a glukancukrazy (GTF-y) katalizują transfer glukozy i uwalniają fruktozę. FTF-y możemy podzielić na lewancukrazy, syntetyzujące lewan (polimer fruktozowy, gdzie monocukry połączone są wiązaniem β(2-6)) i inulocukrazy, syntetyzujące inulinę (polimer fruktozowy zawierający głównie wiązania β(2-1)). Zarówno lewan jak i inulina mogą posiadać rozgałęzienia ale zazwyczaj jest to tylko kilka procent wszystkich wiązań obecnych w cząsteczce polimeru. Generalnie więc obydwa rodzaje enzymów syntetyzują te same typy wiązań tylko w różnych proporcjach (patrz **Chapter 1**).

Lactobacillus reuteri szczep 121 (Lb121) jest przedstawicielem BM i posiada zdolność do produkcji dużej ilości EPS, jeśli hoduje się go w medium zawierającym sacharozę. Jedna trzecia syntetyzowanego polimeru to polimer glukozowy (reuteran), a dwie trzecie to lewan. Dodatkowo Lb121 produkuje śladowe ilości krótkich cząsteczek inuliny. Analiza genetyczna wykazała, że Lb121 posiada dwa geny kodujące

fruktancukrazy. Obydwa geny zostały sklonowane w *Escherichia coli* co umożliwiło ich nadekspresję, oczyszczenie obydwu białek i charakteryzację ich aktywności. Pierwszy z enzymów, lewancukraza (Lev), syntetyzuje lewan o masie cząsteczkowej Mr = 20000 (97%) i 3-4 x 10^6 (3%) oraz niewielkie ilości krótkich fruktooligosacharydów (FOS). Obydwa enzymy były bardzo podobne na poziomie sekwencji aminokwasowej (56 % identyczności i 86 % podobieństwa na odcinku 768 aminokwasów). Wysokie podobieństwo struktury w połączeniu z dużymi różnicami aktywności zrodziły pytanie o strukturalne uwarunkowania różnic w specyfice substratowej i typie katalizowanej reakcji.

Enzymy hydrolizujące wiązania glikozydowe zostały sklasyfikowane jako glikozylohydrolazy (GH) i podzielone na około 100 różnych rodzin (patrz: http://afmb.cnrs-mrs.fr/CAZY/). Inu i Lev z Lb121 należą do rodziny GH68, która zawiera prawie 50 białek (do czerwca 2005) różnego pochodzenia. Ich cechą wspólną jest zdolność do hydrolizowania wiązań β-glikozydowych przy zachowaniu konfiguracji węgla anomerycznego (stąd nazwa – enzymy β-retencyjne). Enzymy te używają tak zwanego mechanizmu Ping Ponga (☺), który polega na tworzeniu, a następnie hydrolizie kowalencyjnego intermediatu enzymu i substratu. Centrum aktywne β-retencyjnych enzymów zawiera przynajmniej dwa aminokwasy katalityczne: pierwszy z nich tzw. nukleofil atakuje centrum anomeryczne cukru i tworzy kowalencyjne wiązanie z resztą cukrową; drugi, tzw. katalizator kwasowo-zasadowy, działa jako kwas w pierwszym etapie reakcji, powodując protonację tlenu tworzącego wiązanie glikozydowe i jako zasada w etapie drugim, kiedy to powoduje deprotonację cząsteczki wody. Często też występuje trzeci aminokwas katalityczny stabilizujący etap przejściowy podczas przebiegu reakcji.

Według ogólnie akceptowanej nomenklatury wiązanie dwucukrów następuje w pozycjach –1 i +1. Numeracja pozycji wiązania zależy od lokalizacji wiązania glikozydowego hydrolizowanego podczas katalizy (zawsze pomiędzy –1 i +1). Aminokwasy w pozycjach –1 i +1 są zawsze położone blisko siebie w prawidłowo sfałdowanym białku. W oparciu o znaną strukturę trzeciorzędową lewancukrazy (SacB) z Gram-pozytywnej bakterii *Bacillus subtilis* aminokwasy tworzące pozycje –1 i +1 w rodzinie GH68 zostały zidentyfikowane. Zakładając, że enzymy FTF zawierają domenę oddziałującą z rosnącym łańcuchem fruktanu, zaproponowaliśmy, że enzymy te zawierają dodatkowe pozycje wiązania cukrów (+2, +3 etc.), które mają duży wpływ na specyfikę substratową i produktową enzymów (patrz **Chapter 4**).

Głównym celem tej pracy doktorskiej była identyfikacja i charakteryzacja cech strukturalnych dwóch FTF-ów z Lb121, mających istotne znaczenie dla ich aktywności i specyficzności wobec produktów reakcji. W niniejszej dysertacji opisujemy (I) identyfikację trzech aminokwasów katalitycznych, (II) lokalizację miejsca wiązania jonów wapnia i termostabilność Inu i Lev, (III) dokładną analizę substratów, produktów i specyficzności obu FTF, (IV) identyfikację cech strukturalnych tych białek, determinujących zakres wielkości syntetyzowanych oligosacharydów i polimerów.

Identyfikacja trzech aminokwasów katalitycznych w enzymach FTF z Lb121.

Aminokwasy katalityczne zostały zidentyfikowane za pomocą porównania sekwencji rożnych białek oraz przy pomocy zlokalizowanej mutagenezy (patrz **Chapter 1**). Porównanie sekwencji Inu, Lev oraz białka o znanej strukturze 3D (SacB), posłużyło do identyfikacji trzech konserwowanych odcinków sekwencji, zawierających idealnie konserwowane reszty kwasowe. Domniemane aminokwasy katalityczne w sekwencji Lev (Asp249, Asp404 oraz Glu503) zostały zmutowane do ich aminowych odpowiedników (Asn lub Gln), a następnie białka zawierające pojedyncze mutacje były wyprodukowane w odpowiednich ilościach w komórkach *E. coli*, oczyszczone i scharakteryzowane. Wszystkie mutanty były praktycznie nieaktywne (przynajmniej 10000 razy mniej aktywne od typu dzikiego (WT) białka), co świadczy o ważnej roli jaką pełnią te aminokwasy w enzymach typu FTF. Największa redukcja aktywności była obserwowana w przypadku mutanta Asp249 (Lev) i Asp272 (Inu). Ta reszta aminokwasowa zlokalizowana jest w odcinku sekwencji konserwowanej we wszystkich enzymach należących do GH68 i została zidentyfikowana jako nukleofil. Na podstawie analizy kinetycznej mutantów Glu503 (Lev) i Glu5236 (Inu) wywnioskowaliśmy, że ta reszta aminokwasowa (i jej odpowiednik u pozostałych członków rodziny GH68) jest generalnym katalizatorem kwasowo/zasadowym. Asp404 (Lev) i Asp424 (Inu), zlokalizowany w konserwowanym motywie Arg-Asp-Pro, pełni rolę stabilizatora etapu przejściowego. Za pomocą dichroizmu kołowego wykazaliśmy, że wprowadzone mutacje spowodowały minimalne zmiany w ogólnej strukturze trzeciorzędowej wszystkich mutantów.

Analiza roli jonów wapnia w enzymach FTF z Lb121 za pomocą mutagenezy.

Analiza znanej struktury trzeciorzędowej białka SacB wykazała, ze zawiera ono miejsce wiązania jonów metalu, najprawdopodobniej wapnia (Ca^{2+}). Chambert i inni, dowiedli ważności jonów wapnia dla prawidłowego fałdowania lewancukrazy z *B. subtilis* w warunkach *in vitro*. W rozdziale 3 (patrz Chapter 3) analizowaliśmy wpływ Ca^{2+} na aktywność i termostabilność Inu i Lev. Dodatkowo skonstruowaliśmy mutanty w przewidywanym miejscu wiązania wapnia i badaliśmy wpływ mutacji punktowych na powinowactwo enzymów do jonów Ca^{2+}. Otrzymane wyniki pokazały że Ca^{2+} odgrywają znaczącą rolę w aktywności Inu i Lev, wpływając na optimum temperaturowe obydwu enzymów oraz ich termostabilność. Dodanie jonów Ca^{2+} do medium reakcyjnego spowodowało zwiększenie aktywności enzymów Inu i Lev w podwyższonych temperaturach. Wyniki te świadczą że obecność jonów Ca^{2+} zapobiega inaktywacji obydwu FTF przez wysoką temperaturę. W celu dokładnego określenia wpływu wywieranego przez jony Ca^{2+} na termostabilność enzymów, zmierzyliśmy aktywności obydwu enzymów w 37°C po uprzedniej inkubacji białek w różnych temperaturach w obecności lub przy braku wapnia. Drastyczny spadek aktywności Inu był obserwowany po inkubacji białka w 40-45°C przez 30 min. Obecność jonów Ca^{2+} w stężeniu 1 mM

zapobiegało nieodwracalnej inaktywacji enzymu. Również termostabilność Lev zależała od obecności jonów wapnia, jednak zależność ta była inna niż w przypadku Inu. Aktywność Lev została drastycznie zredukowana w wyższych temperaturach. Enzym ten w znacznie mniejszym stopniu niż Inu ulegał nieodwracalnej dezaktywacji. Analiza mutantów Inu i Lev w przewidywanym miejscu wiązania wapnia wykazała, że Asp520 (Inu) i Asp500 (Lev) są bardzo istotne dla wiązania jonów Ca^{2+}. Zmutowane białka, w których reszty Asp zostały zastąpione przez Asn lub Ala, wykazywały znaczące obniżenie aktywności w szerokim zakresie badanych temperatur. Stymulujący efekt jonów Ca^{2+} w przypadku mutantów Asp/Asn był znacznie mniejszy niż obserwowany dla typu dzikiego białka i praktycznie nie był obserwowany w przypadku mutantów Asp/Ala. Mutanty Asp/Asn były wciąż zdolne do wiązania jonów $Ca^{2+,}$ ale oddziaływanie to było bardzo słabe. Powinowactwo zmutowanego wariantu Inu (Asp520Asn) do Ca^{2+} zostało znacznie zredukowane (ok. 1600 razy), a powinowactwo zmutowanej wersji Lev zmniejszyło się tylko 35-krotnie.

Przedstawione dane dowodzą, że jony Ca^{2+} odgrywają znaczącą rolę w stabilizowaniu struktury Inu i Lev. Obydwa enzymy posiadają miejsce wiązania wapnia wykazujące wysokie powinowactwo do jonów Ca^{2+}, w którego skład wchodzi min. reszta Asp zlokalizowana we fragmencie sekwencji konserwowanym we wszystkich enzymach FTF, pochodzących z bakterii Gram-ujemnych. Ta reszta aminokwasowa nie występuje w enzymach pochodzących z bakterii Gram-ujemnych, sugerując że enzymy te nie mają zdolności wiązania jonów Ca^{2+}. Niedawno została opublikowana pierwsza struktura 3D enzymu typu FTF, pochodzącego z bakterii Gram-ujemnej (*Gluconacetobacter diazotrophicus*), potwierdzając nasze przewidywania.

Transglikozylacja katalizowana przez enzymy FTF z Lb121.

Szczegółowa analiza produktów reakcji katalizowanych przez Inu i Lev wykazała podobieństwa jak i różnice pomiędzy tymi dwoma enzymami (patrz Chapter 4). Głównym produktem syntetyzowanym przez Lev był lewan o dużej masie cząsteczkowej, podczas gdy Inu syntetyzował głównie fruktooligosacharydy i niewielkie ilości polimeru – inuliny. Enzym Inu syntetyzował głównie małocząsteczkowe FOS poprzez uwalnianie intermediatów reakcji po każdorazowym przedłużeniu łańcucha cukrowego (mechanizm nieprocesywny). Charakterystyczny wzór syntetyzowanych oligosacharydów (przypominający szczeble drabiny) obserwowany przy zastosowaniu metody chromatografii cienkowarstwowej (TLC), świadczy o wysokim powinowactwie tego enzymu do krótkich łańcuchów oligosacharydowych. Jednocześnie, w porównaniu do Inu, Lev syntetyzował niewielkie ilości FOS i zdecydowanie więcej polimeru. Otrzymany wynik sugeruje, że Lev katalizuje procesywny typ reakcji, gdzie rosnące łańcuchy polimeru pozostają połączone z cząsteczkami enzymu podczas reakcji i są uwalniane dopiero po osiągnięciu względnie dużych rozmiarów. Ponieważ osiem reszt aminokwasowych, tworzących centra aktywne tych enzymów jest identyczne, zaproponowaliśmy, że FTF posiadają dodatkowe miejsca wiązania substratu (+2, +3 itd.)

różne dla Inu i Lev, które powodują powstanie różnic w strukturze obydwu enzymów, a przez to różnice w typie katalizowanych reakcji.

Inu i Lev z Lb121 mogą używać substratów innych niż sacharoza, np.: rafinozy i oligosacharydów typu inuliny (zawierających wiązania β(2-1)) składdających się z 3 do 5 reszt cukrowych (stopień polimeryzacji 3 do 5). Analiza produktów reakcji przy użyciu TLC wykazała, że Inu utylizuje takie substraty wydajniej niż Lev. Głównymi produktami reakcji z użyciem takich substratów były oligosacharydy zawierające o jedną resztę cukrową więcej lub o jedną mniej. Te dane sugerują, że obydwa enzymy katalizują reakcję dysproporcjononacji. Jednocześnie, żaden z enzymów nie syntetyzował cząsteczek polimeru przy użyciu tych substratów. W przypadku Inu brak syntezy polimeru był obserwowany nawet wtedy, gdy sacharoza była używana jako substrat, co może być wytłumaczone małym powinowactwem tego enzymu do rosnących cząsteczek polimeru. Brak polimeryzacji katalizowanej przez Lev przy użyciu substratów dłuższych od sacharozy sugeruje, że dla przeprowadzenia tego typu reakcji enzym wymaga cząsteczki dwucukru związanego w pozycji –1 i +1służącego jako donor. Użycie przez Lev dłuższych cząsteczek oligomerów cukrowych, jako donorów, wymaga aby dalsze miejsca wiązania akceptora (+2, +3 itd.) były zwalniane po każdorazowym przedłużeniu cząsteczki akceptora i przez to jedynie nieprocesywny typ reakcji może mieć miejsce.

Synteza polimeru/oligosacharydów przez enzymy FTF.

Rozdział 5 (Chapter 5) to próba wyjaśnienia roli trzech konserwowanych reszt aminokwasowych dla aktywności i typu produktów syntetyzowanych przez fruktancukrazy. Te trzy aminokwasy zostały zmutowane w Inu, po czym otrzymane mutanty zostały scharakteryzowane biochemicznie. W oparciu o dane strukturalne enzymu SacB, Trp340 w sekwencji Inu został zidentyfikowany jako reszta aminokwasowa „zamykająca" miejsce wiązania sacharozy. Mutant Inu zawierający mutację punktową Trp340Asn był pozornie nieaktywny (tylko 0,5 % aktywności typu dzikiego białka) co potwierdziło znaczącą rolę tego aminokwasu w miejscu aktywnym białka. Podobny spadek aktywności był obserwowany w przypadku mutanta Arg423His (0,3% aktywności WT), prawdopodobnie wskutek zaburzeń w bardzo precyzyjnej sieci oddziaływań stworzonych przez odpowiednią resztę aminokwasową w SacB.

Aktywność mutanta Inu Arg423Lys nie została tak drastycznie zmniejszona w porównaniu do WT białka. Jednocześnie mutant ten syntetyzował inne oligosacharydy niż WT jeśli sacharoza była używana jako substrat. W porównaniu do WT mutant ten syntetyzował mniej FOS i zdecydowanie więcej polimeru. Podobna zmiana aktywności enzymu była obserwowana w przypadku mutanta Inu Trp271Asn. Interesujący jest fakt, że obydwa aminokwasy (Arg423 i Trp271) są zlokalizowane na dnie centrum aktywnego enzymu i tworzą pozycję –1 dla wiązania substratu. W oparciu o przedstawione dane zasugerowaliśmy, że pozycja –1 jest ważna nie tylko dla rozpoznania substratu lecz także determinuje typ syntetyzowanego produktu reakcji.

Podsumowując, w niniejszej dysertacji przedstawiamy identyfikację trzech katalitycznych reszt aminokwasowych w enzymach FTF z Lb121. Taka analiza właściwości mutantów połączona z dokładnym pomiarem ich aktywności została opublikowana po raz pierwszy.

Asp520 (Inu) oraz odpowiadający mu Asp500 (Lev) to reszty aminokwasowe, odgrywające ważną rolę w stabilizacji struktury obydwu enzymów i kluczowe dla wiązania jonów wapnia. Mutacje wprowadzone w tej pozycji w sekwencji Inu i Lev spowodowały znaczące zmiany w zależności aktywności obydwu enzymów od temperatury oraz w termostabilności tych białek. Reszty aminokwasowe, tworzące miejsce wiązania wapnia, są konserwowane we wszystkich enzymach należących do rodziny GH68 i pochodzących z bakterii Gram-dodatnich. Wydaje się, że miejsce wiązania wapnia w enzymach pochodzących z Gram-ujemnych bakterii i należących do GH68 nie występuje.

Obydwie fruktancukrazy z Lb121 (Inu i Lev) są bardzo podobne na poziomie sekwencji aminokwasowej, ale katalizują różne reakcje tranglikozylacji. Inu syntetyzuje głównie fruktooligosacharydy, podczas gdy głównym produktem reakcji Lev jest polimer. Te różnice są prawdopodobnie spowodowane przez inną organizację miejsc wiązania +2, +3 i dalszych w obydwu enzymach.

W końcu pokazaliśmy, że mutacje wprowadzone w miejscu wiązania fruktozy (pochodzącej z naturalnego substratu – sacharozy) wpływają na wielkość syntetyzowanych fruktanów. Otrzymanie struktury 3D dwóch enzymów FTF, pochodzących z Lb121, zawierających cząsteczkę sacharozy związaną w centrum aktywnym enzymów i / lub większą cząsteczkę oligosacharydu (lub polisacharydu) w centrum wiązania akceptora, powinno przyczynić się do wyjaśnienia dlaczego tak podobne cząsteczki enzymu katalizują tak różne reakcje. Koniec.

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